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STUDIES ON HISTIDINE RESIDUES IN HEMEPROTEINS RELATED TO THEIR ACTIVITIES

I. CARBOXYMETHYLATION OF HEMOGLOBIN WITH BROMOACETIC ACID

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(Received for publication, January 6, 1960)

Considering the pK values and heats of ionization of heme-linked acid groups, Wyman (*1*, *2*) and Pauling *et al.* (*3*, *4*) concluded that in hemoglobin each of the heme-irons is linked with one of imidazole groups of histidine residues respectively. It was suggested by Conant (*5*) that another imidazole group is also weakly linked with each of the heme-irons, at the opposite site, through its acid group representing pK_1.

It was shown by Banaschak and Jung (*6*) that the absorption spectrum of methemoglobin treated with urea was distinguished at the Soret region after addition of dithionite from that of native hemoglobin, and modification of protein moiety was indicated. The present author noticed that the solution of once lyophilized hemoglobin showed a slight decrease in absorption at 500 to 600 mμ as compared with that of native hemoglobin, but there was little change at the Soret region (Fig. 1). It appears that the anhydro-type hemoglobin (*7*) was formed at first by lyophilization which led to a slight modification of the hemoglobin molecule and consequently resulted in a change of the absorption spectrum.

It has been reported that haloacetic acids react with histidine, tyrosine, lysine and cysteine residues in proteins (*8–10*). Since histidine residues seemed to act an important role as heme-linked groups in the secondary structure of hemoglobin, the reactivity of histidine residues in native, urea-denatured and lyophilized hemoglobins toward haloacetic acids were compared, and changes of the absorption spectra by carboxymethylation were examined.

The results showed differences in reactivity of histidine residues between native and urea-modified hemoglobins, whereas lyophilized hemoglobin was carboxymethylated at the same rate as the native one.

It was then concluded that the excess histidine residues which reacted with bromoacetic acid after treatment with urea were masked in native state, and might be related with a secondary structure of hemoglobin. On

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the other hand, a minute modification after lyophilization seemed to have no effect upon the states of histidine residues in the molecule.

EXPERIMENTAL

1. Preparation of Hemoglobin—Crystalline hemoglobin was prepared from fresh bovine erythrocytes by alcohol fractionation at 0 to 2° and recrystallized six times from an aqueous alcohol solution by decreasing the alcohol concentration gradually to below 20 per cent. A portion of the sample was lyophilized with a freeze drying machine (Model RH 1000, laboratory of Kyowa-Oyobutsuri).*

2. Haloacetic Acids—Bromo- and iodo-acetic acids were obtained commercially and purified by vacuum distillation.

RESULTS

1. Carboxymethylation of Hemoglobin—For the determination of the optimum pH of the reaction, approximately 1 per cent hemoglobin solution was adjusted to each described pH in Table I with *N*/10 NaOH or HCl, and also 1*M* of haloacetic acids solutions were adjusted to the corresponding pH by adding MgO. After adjusting the pH, the carboxymethylation was carried out with each 8 ml. of the hemoglobin of different pH values and 2 ml. of the bromo- or iodo-acetic acid solution of a corresponding pH in the presence of MgO at 30°. After 4, 8 and 24 hours of incubation, 1 ml. of each reaction solution was taken and centrifuged. The supernatant solution was then hydrolyzed with 5.5 *N* HCl for 10 hours at 115°. Each of the hydrolyzed solutions was distilled under evacuation to remove HCl and diluted to 10 ml. with distilled water. Histidine was determined by Hunter's method (*11*) modified by Narita *et al.* (*12*). To 2.0 ml. of the

TABLE I

Carboxymethylation of Hemoglobin with Bromo- and Iodo-Acetic Acids

	pH	4 Hours		8 Hours		24 Hours	
		His (%)	Tyr (%)	His (%)	Tyr (%)	His (%)	Tyr (%)
Br-Acetic Acid	2.8	96	100	90	98	82	97
	5.6	95	100	88	98	81	98
	9.7	80	100	65	97	30	95
	11.8	88	100	79	97	37	95
I-Acetic Acid	2.8	88	99	74	98	49	95
	7.4	90	99	85	97	75	95
	9.5	78	99	54	98	30	94

hydrolyzed protein solution, were added 0.25 ml. of 2 *N* H_2SO_4, and then a few

* By courtesy of Dr. G. Funatsu, Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University.

drops of $N/10$ $KMnO_4$ solution until the light pink color of excess $KMnO_4$ disappeared between 30 to 50 seconds. The distilled water was then added to a final volume of 2.75 ml. The procedure thereafter was carried out with a half scale of Hunter's method. Tyrosine was determined by Folin's phenol reagent (*13*), taking 0.1 ml. of the hydrolyzed solution, and the procedure was carried out at 1/10 scale of the original method. The N concentration of hydrolyzed solution was determined by the micro-Kjeldahl method (Table I).

2. *Carboxymethylation of Native and Lyophilized Hemoglobin*—The carboxymethylation of native and lyophilized hemoglobin was performed at pH 9.0 in the presence of MgO, the conditions being judged from the results in Table I. Eight ml. of hemoglobin solution containing about 75 mg. protein (pH 9.0) were treated with 1 ml. of an aqueous solution of 2*M* bromoacetic acid (pH 9.0 with MgO suspension). The rate of carboxymethylation was measured by determining diazocoupling groups, using Macpherson's method (*14*), since the decrease in diazocoupling groups during carboxymethylation was considered to be due mainly to decrease in the histidine groups (Table I), and the method was much more convenient than Hunter's method. After a given time described in Table II, 0.5 ml. of each reaction mixture was centrifuged, and 0.25 ml. of the supernatant was taken and diluted to 10 ml. with distilled water. The following procedure was carried out at a 1/2.5 scale of the Pauli's method of Macpherson, using 4 ml. of the diluted solution (Table II).

The rate of carboxymethylation was not much different between native and lyophilized hemoglobins. The reaction was completed within 10 hours.

TABLE II

Decrease in Diazo-Coupling Groups during Carboxymethylation of Hemoglobin

Reaction Time (hours)	I[1]		II[2]	
	O.D. 530 mμ	%	O.D. 530 mμ	%
0	1.220	100	1.425	100
1	1.115	91.5	1.320	92.9
2	.995	81.5	1.170	82.2
4	.835	68.4	.975	68.4
6	.730	59.8	.825	58.0
8	.650	52.8	.745	52.3
10	.580	47.5	.675	47.4
20	.550	45.0	.650	45.6
24	.550	45.0	.650	45.6

1) Native hemoglobin 2) Lyophilized hemoglobin.

3. *Carboxymethylation of Urea Denatured Hemoglobin*—Each portion of 5 ml. of 1 per cent hemoglobin solution was incubated in the presence of 2, 6 and

8 *M* concentration of urea, respectively, at 30° over night. The reaction solutions were used for two separate experiments, *i. e.*, determinations of absorption spectrum and the rate of carboxymethylation. To determine the absorption spectrum, 1 ml. of each solutions was taken and diluted with *M*/10 phosphate buffer of pH 6.8 (the final concentration of the buffer solution was made to *M*/40), so that the reading of the absorbancy was appropriate at different wave lengths.

The absorption spectra were measured by a Beckman Model DU. The concentration of protein was calculated by determining N content of the original hemoglobin solution (Fig. 1).

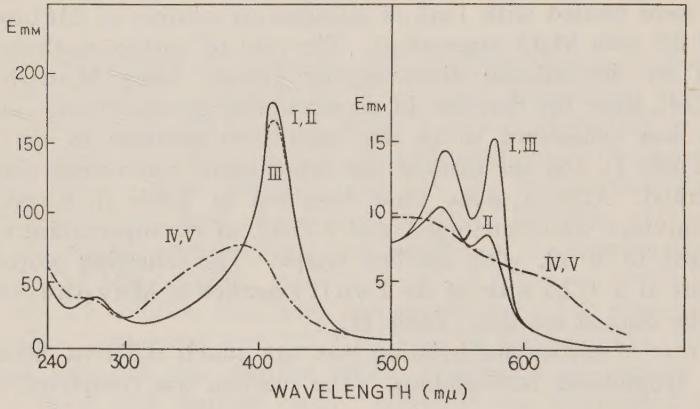

Fig. 1. Absorption spectra of native, lyophilized and urea denatured hemoglobins. Curve I: Native, II: Lyophilized, III: 2 *M* Urea denatured,[1] IV: 6 *M* Urea denatured and V: 8 *M* Urea denatured.

1) Duration of urea treatment: 12 hours

The carboxymethylation was performed in the presence of urea directly after the incubation process, since the removal of urea from the incubation mixture made the hemoglobin insoluble in water and neutral salt solution. The other reaction conditions were the same as previously mentioned. To each portion of 4 ml. of the 2, 6 and 8*M* urea denatured hemoglobin solutions, 0.5 ml. of 2*M* of bromoacetic acid was added respectively. After 24 hour incubation at 30°, each of the reaction mixtures was centrifuged and 1 ml. was used for measurement of the absorption spectra (Fig. 2).

The determination by Pauli's method was inhibited by urea, therefore each 3 ml. of the reaction solution were transfered into cellophane tubes and dialyzed in a cold place (0–5°) for a week, and the histidine content of the solution was determined after hydrolysis as described above (Table III).

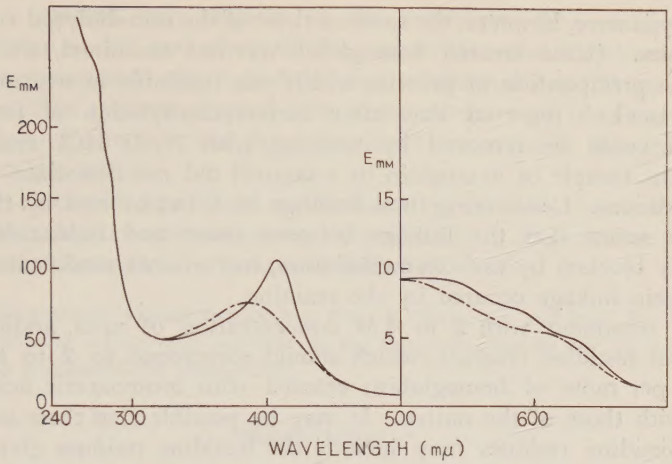

FIG. 2. Absorption spectra of carboxymethylated hemoglobin.
—— CM[1]-Hemoglobin, —·—·— CM-Urea-denatured hemoglobin.

1) Carboxymethylated.

TABLE III

Carboxymethylation of Urea Denatured Hemoglobin

Remaining histidine and tyrosine residues were determined after 24 hours reaction of carboxymethylation of native and urea denatured hemoglobin.

Urea (*M*)	His (%)	Tyr (%)
0	44	95
2	37	95
6	20	95
8	19	92

DISCUSSION

With respect to the reaction mechanism of histidine residues in hemoglobin with bromoacetic acid, it is considered that histidine residues which were not carboxymethylated were undissociated and their imino hydrogen atoms were probably bounded with some groups internally in the protein, or were masked in the molecule on account of a steric hindrance, since usually imino H of histidine is expected to be dissociated at pH 9 and bromoacetic acid reacts as an electrophilic substance. After carboxymethylation of native and lyophilized hemoglobins, each of the reaction mixture was dialyzed against distilled water at 5°, and each absorption spectrum was measured. The absorption spectra of carboxymethylated hemoglobins

after dialysis were, however, the same as these of the non-dialyzed corresponding samples. (Urea treated hemoglobin was not examined, since dialysis caused the precipitation of protein, which was insoluble in neutral salt solution). Clarke* reported that after carboxymethylation of hemoglobin, porphyrin could be removed by washing with $N/20$ HCl and acetone, whereas the sample of hemogloin in a control did not dissociate under the same conditions. Considering these findings by Clarke and by the present author, it seems that the linkage between heme and imidazole was not completely blocked by carboxymethylation, but a weak modification around heme-protein linkage occured by the reaction.

After treatment with 2 to 8 M concentration of urea, additional 7 to 22 per cent histidine residues (which should correspond to 2 to 8 moles of histidine per mole of hemoglobin) reacted with bromoacetic acid in comparison with those of the native. It may be possible that these additionally reacted histidine residues may include the histidine residues giving pK_1 as defined by Conant (*5*), for the protein might be modified more extensively in the presence of urea and hence histidine residues which initially were weakly linked with some groups in the hemoglobin molecule might become more reactive with bromoacetic acid.

The absorption spectra at the visible region of urea denatured hemoglobin, and of carboxymethylated native and urea denatured hemoglobins were shown to be similar to those obtained with methemoglobin as reported by Jung *et al.* (*6*) concerning the effect of urea on methemoglobin. The remarkable decrease in the absorbancies at the visible region after carboxymethylation of native and 2 M urea treated hemoglobins might be due to the intensified effect of bromoacetic acid together with that of urea itself on the formation of methemoglobin. The bromine liberated during carboxymethylation would act as an oxidant to heme iron.

However, there is a possibility that the observed big change in the absorption spectrum at the Soret region after carboxymethylation of 2 M urea-modified hemoglobin may be resulted, at least in part, by a modification around the linkage between heme and protein, since it is assumed that the absorbancy at the Soret region is dependent upon either heme itself and the linkage between heme and protein. The proposed possibility could be supported by the following findings: (i) different hemeproteins having same numbers of protoheme give different molar extinction coefficients, and (ii) in comparison to native hemoglobin, urea modified methemoglobin showed a distinguished decrease in absorbancy at the Soret region after being reduced with dithionite (*6*).

SUMMARY

1. Native, lyophilized and urea denatured hemoglobins were carboxymethylated with bromoacetic acid in the presence of MgO. The optimum pH of the reaction to attack histidine residues in hemoglobin was pH 9,

* Clarke, H. T., personal communication.

and tyrosine reacted only slightly at this condition. The reaction ended within 10 hours at 30° and about 60 per cent of the total histidines reacted.

2. After treatment with 2 to 8 *M* urea, additional 7 to 22 per cent of the total histidine residues reacted with bromoacetic acid in comparison to those with native one, whereas the lyophilized hemoglobin reacted in the same way as the native.

3. The absorption spectrum at the Soret region was remarkably diminished by the carboxymethylation and particularly a big change was observed with the hemoglobin denatured with 2 *M* urea.

The author wishes to express gratitude to Prof. Y. Oshima and Prof. M. Funatsu for their invaluable advice and criticism during this work, and to Mr. S. Abe for assistance in the preparation of hemoglobin.

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STUDIES ON HISTIDINE RESIDUES IN HEMEPROTEINS RELATED TO THEIR ACTIVITIES

II. CARBOXYMETHYLATION OF CATALASE WITH BROMOACETIC ACID

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(Received for publication, January 6, 1960)

Using Weil's method of histidine specific photooxidation in the presence of methylene blue (*1*), it was shown that histidine residues in catalase were destroyed and catalase decreased in its enzymatic activity.** The results seemed to indicate an important role of the histidine on catalase activity, relating to a specific configuration. In the previous paper (*2*), carboxymethylation of hemoglobin was reported, and a part of histidine residues were shown to be masked in the native state and probably related to its secondary structure. In the present study catalase was carboxymethylated in the presence and absence of urea, and reactivities of histidine residues toward bromoacetic acid were compared. The changes of catalase activity and absorption spectrum were also examined.

EXPERIMENTAL

1. Preparation of Crystalline Catalase—The crystalline catalase was prepared from a fresh bovine liver by the method of Shirakawa (*3*), and recrystallized several times. The crystalline catalase obtained was shown to be nearly homogeneous (*4*) by the column chromatography with calcium phosphate gel prepared by the method of Tiselius *et al.* (*5*).

2. Assay of Catalase Activity—An aqueous solution of catalase was diluted to a concentration of approximately 10 μg. per ml. with $M/15$ phosphate buffer of pH 6.8, and the catalase activity was determined by Euler-Josephson's method (*6*). The concentration of catalase was determined by measuring the N content using the micro-Kjeldahl method. Taking the N content in catalase as 16.8 per cent (*7*), the K_f value of the crystalline sample was calculated as 32,000.

3. Measurement of Absorption Spectrum—The absorption spectra of catalase, and carboxymethylated native and urea denatured catalases were measured using a Beckman model DU spectrophotometer. All measurements were made in $M/40$ phosphate buffer of pH 6.8.

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** Nakatani, M., unpublished data.

RESULTS

1. Carboxymethylation of Catalase—Following the optimum conditions of carboxymethylation of histidine residues in hemoglobin (*2*), catalase was carboxymethylated at pH 9.0 with bromoacetic acid in the presence of MgO. Catalase solution of approximately 1 per cent was adjusted to pH 9.0 with *N*/10 NaOH, and 2 *M* bromoacetic acid solution was also made to pH 9.0 with MgO. Then, 4 ml. of the catalase solution were incubated with 1 ml. of the bromoacetic acid solution. The reaction was carried out at 30°, and the pH of the solution was carefully adjusted to 9.0 during the reaction by the addition of MgO, since the catalase activity decreases rapidly above this pH. After periods of incubation as described in Table I, each 0.5 ml. of the solution was taken and centrifuged, and the decrease in the diazocoupling groups was determined by Pauli's method of Macpherson (*8*). From the supernatant, 0.2 ml. was taken and diluted to 10 ml. with distilled water, and 4 ml. of the diluted solution were used for the determination of the diazocoupling groups (Table I).

TABLE I

Effect of Carboxymethylation on Catalase Activity and Decrease of Diazocoupling Groups

Reaction Time (hours)	Catalase Activity k (%)		Diazocoupling Groups (%)
	MgO	MgO+ Br-Acetic acid	
1	95	94	90
2	94	92	85
3	92	89	82
5	91	85	77
7	90	82	73
10	89	79	69
15	89	75	66
20	88	72	65
24	88	70	65

2. Carboxymethylation of Urea Treated Catalase—The ratio of decreases in intact histidine residues and enzymatic activity after carboxymethylation of catalase molecule was found to be different from that obtained after photooxidation; about half of the activity was lost when the histidine residues were reduced to 50 per cent by the photooxidation, whereas about 20 per cent of the activity was lost by 45 per cent decrease in histidine by carboxymethylation. The photochemical action might decompose histidine residues at random, whereas bromoacetic acid might attack only the dissociable and unmasked histidine residues. It was considered that catalase

activity are not necessarily dependent on all histidines in the molecule, but mainly on those which are related to some specific structure in the molecule. Many of the histidine residues which reacted with bromoacetic acid might not be directly related with the specific structure of catalase.

Catalase was treated with urea to detect histidine residues which might be masked at the native state. A solution of catalase in $M/10$ phosphate buffer of pH 6.8 was incubated in the presence of 1.75, 3.5 and 5 M con-

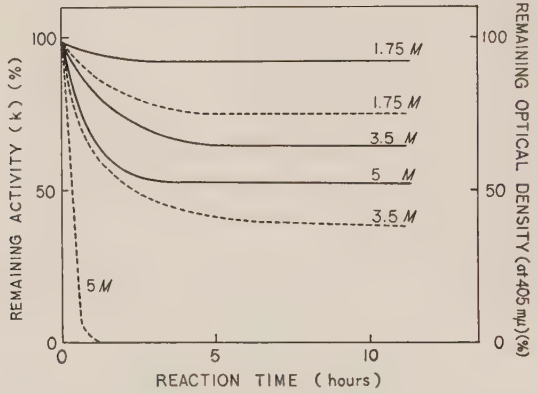

Fig. 1. Effect of urea on catalase activity and absorbancy at the Soret band.
—— Optical Density, ----- Activity.

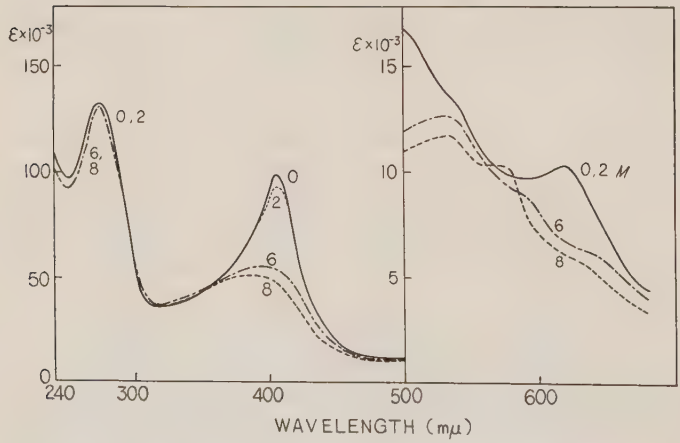

Fig. 2. Effect of urea on the absorption spectrum of catalase.
—— Native Catalase, ----- 2 M Urea denatured,
—·—· 6 M Urea denatured, — — — — 8 M Ured denatured.

centrations of urea respectively, at 30° and decreases in the enzymatic activity as well as in absorbancy at 405 mμ were measured (Fig. 1). By incubation with urea, enzymatic activity and absorbancy at 405 mμ decreased generally within 10 hours.

Each 5 ml. of the aqueous solution of catalase containing approximately 50 mg. of the protein were adjusted to pH 6.8 with $N/10$ NaOH, and incubated at 30° in the presence of 2, 6 and 8 M urea over night respectively. The urea-modified catalase solutions were then examined for the absorption spectrum: 1 ml. of each solution was diluted to 5 ml. with phosphate buffer of pH 6.8 as to give the final buffer concentration of $M/40$ (Fig. 2). Characteristic decreases in the absorption at 405 mμ were observed with 6 and 8 M urea, whereas the decrease after the 2 M urea treatment was only slight.

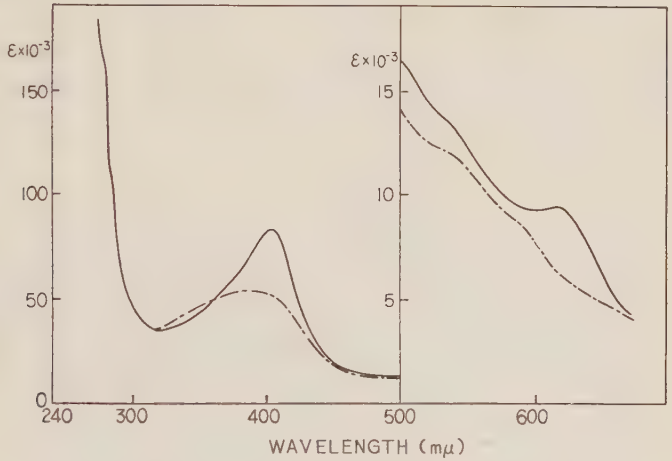

FIG. 3. Absorption spectra of carboxymethylated catalase.
—— CM-Catalase, —·—· CM-Urea-denatured catalase.

TABLE II

The Effect of Urea Denaturation on the Carboxymethylation of Histidine Residues in Catalase

Urea (M)	His Remaining	
	per cent	moles
0	45	42.8
2	34	32.2
6	26	24.7
8	25	23.7

The urea-modified catalase solution was adjusted to pH 9.0 with MgO, and 4 ml. of which were used for carboxymethylation in the presence of urea, since the removal of urea made the catalase insoluble in water and in neutral salt solutions as observed in the case of hemoglobin. Other conditions of the reaction were the same as described in the preceding

section. After 24 hours of reaction, each reaction mixture was centrifuged and 1 ml. of supernatant was taken and used for the measurement of the absorption spectrum (Fig. 3).

Then, the remaining 3 ml. of the reaction mixture were transfered into a cellophane tube and dialyzed against distilled water in the cold (0–5°) over a week, the outer solution being replaced. The dialyzed catalase solutions were then taken and hydrolyzed with 5.5 *N* HCl at 115° for 10 hours. The histidine content was determined by Hunter's method (*9*) modified by Narita *et al.* (*10*). The nitrogen concentration in the hydrolyzed solution was determined by micro-Kjeldahl method and protein concentration was calculated (Table II).

The histidine content in catalase was determined to be 6.0 per cent which corresponds to approximately 95 moles per mole of catalase, adopting its molecular weight to be 248,000 (*11*).

DISCUSSION

Assuming that there are 95 moles of histidine per mole of catalase, about 10 to 20 moles more histidines were found to become reactive by the treatment with urea in comparison to the control experiment without urea. In spite of that the catalase molecule is larger than the hemoglobin molecule, the ratio of carboxymethylated histidines in catalase was similiar to that of hemoglobin in the absence of urea (approximately 55 per cent of the histidine was carboxymethylated). However, different ratio of carboxymethylated histidine residues with the two proteins were observed after treatment with urea. With hemoglobin, 7 to 22 per cent (2 to 8 moles) more of the histidines reacted with the bromoacetic acid after treatment with 2 to 8 *M* urea, whereas in case of catalase 11 to 20 per cent (10 to 19 moles) more reacted above those obtained without urea treatment. The different rates of reactivity of histidine residues might be due to the different state of imidazole N in two proteins. The histidine which did not easily react might be masked in the native state by a steric hindrance and possibly a part of them might be bound with some groups internally in the protein. After the treatment with 8 *M* urea, 25 per cent histidine residues still remained without affected by carboxymethylation. Some of these uncarboxymethylated histidines might be tightly bound internally in the protein, though not conclusively verified as yet.

By treating with urea of 6 and 8 *M*, the absorption spectrum of catalase decreased remarkably at regions of 405 mμ and 500 to 600 mμ, whereas with 2 *M* of urea only a slight change was observed. However, the carboxymethylation of the 2 *M* urea-denatured catalase resulted in a significant decrease in the absorbancies at the mentioned regions and a pattern of the absorption spectrum similiar to those of 6 or 8 *M* urea-treated catalase was obtained. On the other hand, the change in the absorption spectrum of the native catalase after carboxymethylation was considerably smaller than that of the urea-treated catalase.

As it is known that the heme-iron of catalase is in a ferric state, the decrease in absorbancies at the mentioned regions can not be attributed to the formation of methemoglobin-type catalase such as discussed in the preceding paper on hemoglobin. It is known that the denaturation by urea or by the hydrolytic action with trypsin and pepsin (*12*) results in similar decreases in the absorption spectrum of catalase. It is probable that the absorbancy at the Soret band may partly depend upon the mode of heme-protein linkage as discussed in the preceding paper (*2*). The unfolding of the protein molecule by urea or by proteolysis may cause the modification of the heme-protein linkage; the groups which weakly linked to heme would be affected through their actions, resulting in a change of the absorbancy.

In the presence of 2 *M* urea, the carboxymethylation of heme linked groups may be promoted by unfolding effect of urea, and this may cause the change of the absorbancy. Thus, the intensed decrease in the absorbancy as observed by carboxymethylation of 2 *M* urea treated catalase could pressumably be brought about by carboxymethylation of the groups which are more or less responsible for the heme-protein linkage.

It was reported by Korman *et al.*, that reactive disulfide linkage were increased during carboxymethylation of albumin (*13*). This suggests that denaturation process might be involved in carboxymethylation of catalase. During the carboxymethylation, bromide is liberated and the bromine may effect the modification of the catalase molecule, even in the presence of MgO which could act as neutralizing agent. This modification would also give rise to the decrease in the absorbancy.

SUMMARY

1. Catalase was treated with bromoacetic acid in the presence of MgO at pH 9. At these conditions, 35 per cent of total diazocoupling groups reacted, and about 20 per cent decrease of the total enzyme activity was resulted.

2. The absorption spectrum changed slightly after the carboxymethylation of the native catalase, whereas 2 *M* urea-denatured catalase showed a remarkable decrease after the carboxymethylation.

3. After treatments of catalase with 2, 6 and 8 *M* urea respectively, additional 11 to 20 per cent of the histidines (10 to 19 moles in one mole of catalase) reacted with bromoacetic acid in comparison with untreated sample. It seems that these urea activated histidine residues are masked in native state, a part of which might be related with secondary structure of the protein.

The author wishes to express her gratitude to Prof. Y. Oshima and Prof. M. Funatsu for their invaluable advice and criticism during the course of this work, and to Mr. S. Abe for his assistance in the preparation of catalase.

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IMMUNOCHEMICAL STUDIES OF INSULIN

II. THE SPECIFICITY OF INSULIN NEUTRALIZING ANTIBODY AND EXPERIMENTAL DIABETES

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Previously, Lewis (*1*) demonstrated that the uterine strips of guinea pigs sensitized with insulin from one species could be responded to insulin from another species with manifestation of anaphylactic contraction. Bernstein, Kirsner and Turner (*2*), Wasserman and Mirsky (*3*) and Arquilla and Stavitsky (*4*) observed the immunological cross reaction among the insulin preparations from the various species, by the different techniques.

Using insulin neutralization procedure, Moloney and Coval (*5*) and Moloney and Goldsmith (*6*) have confirmed the same facts. They also found that the guinea pig antisera react with endogenous mouse insulin and they were able to produce diabetes in the mouse by the injection of these antisera.

In the present paper, the specificity of antibodies obtained from guinea pig immunized with ox insulin has been studied by the neutralizing technique and skin test. Also, the experimental diabetes induced by the insulin neutralizing antibodies in the mouse has been investigated.

MATERIALS AND METHODS

Insulin—Whale (*Finback* and *Sperm*), ox and pig crystalline insulin were kindly given by Dr. T. Sawada, 3rd Department of Internal Medicine, Faculty of Medicine, Kyushu University and Dr. T. Shibata, Taiyo Fishery Co. Ltd., and Novo's Lente Insulin and crystaline pig insulin by Dr. Y. Nojima, the Kodama Shoji Co., and bonito insulin by Dr. T. Okuyama, Department of Chemistry, Faculty of Science, Tokyo Prefectural University.

Crude insulin extracts from rabbit, guinea pig and dog were prepared according to the method described by the Goldsmith and Moloney (*7*).

Preparation of Anti-Insulin Sera—Guinea pigs were immunized with ox insulin by the method as previously reported (*8–9*).

The insulin neutralizing activity of antiserum was measured according to the method as previously described (*8–9*).

Blood Sugar Determination—Mice were bled from the jugular vein and rabbit from the ear vein. Blood sugar estimations were done by the method of Hagedorn-Jensen.

Preparation of 50 Per Cent Ammonium Salfate Precipitated-Fraction of Guinea Pig Immune Serum—Guinea pig immume serum was added with an equal volume of saturated ammonium sulfate solution and the resulting precipitates were washed with 55 per cent $(NH_4)_2SO_4$ solution twice and resolved with distilled water and dialysed against diluted buffered saline in the cold room. The dialysate was lyophilized and stored in a desiccator at the cold temperature. The lyophilized powder was dissolved with buffered saline as needed.

As a buffered saline, phosphate buffer (0.15 *M* Na_2HPO_4-HCl pH 7.4) combined with an equal volume of 0.9 per cent NaCl was used.

RESULTS

The Specificity of Insulin Neutralizing Activity of Guinea Pig Antisera—The insulin preparations from various species were tested by a mouse convulsion procedure. As shown in Table I, guinea pig immune serum could neutralize not only ox insulin used as an antigen but also pig, whale, dog, rabbit and bonito insulin. On the other hand, no neutralization by the immune serum was observed for guinea pig insulin which was called native insulin by Moloney and Coval (*5*).

TABLE I

Insulin Neutralizing Specificity of Guinea Pig Immune Serum

Insulin		Neutralization occured
Ox	Crystalline	+
	Amorphous	+
Pig	Crystalline	+
Whale	Crystalline	+
Finback	Crystalline	+
Sperm	Crystalline	+
Bonito	Amorphous	+
Dog	Crude	+
Rabbit	Crude	+
	Endogenous	+
Mouse	Endogenous	+
Guinea	Crude	−
	Endogenous	−

Guinea pig immune serum was prepared using ox insulin as an antigen. The criteria for neutralization were prevention of convulsion due to each insulin tested. The mixture of 0.25 ml. of immune serum and 0.25 units of insulin was injected to a group of five mice. Other criteria for neutralization were elevation of blood sugar level of animal injected by immune serum (See Fig. 1 and 2).

Next, it was tested whether the endogenous insulin of rabbit, mouse and guinea pig is neutralized by guinea pig immune serum. The intra-

venous injection of immune serum into rabbit was followed by an elevation of blood sugar level as shown in Fig. 1 (curve a), but injection of normal serum did not cause any change of blood sugar level (curve c). When the mixture of immune serum and insulin was injected into rabbit intravenously, the elevation of blood sugar level rather than its lowering due to insulin was observed (curve b).

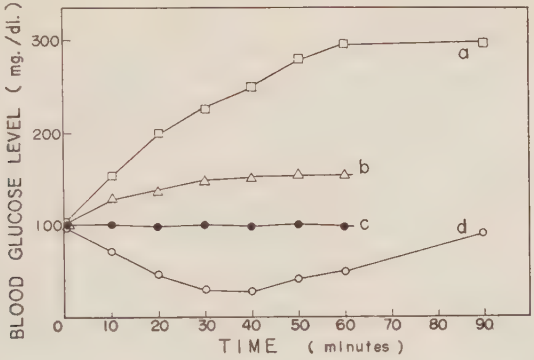

FIG. 1. Elevation of blood glucose level in rabbit following injection of guinea pig immune serum.

a. 1 ml. of immune serum (neutralizing activity : 2 ml.)
b. 0.5 ml. of immune serum plus 0.5 U. of whale insulin
c. 1 ml. of normal serum
d. 0.5 U. of whale insulin

Each was injected intravenously to rabbit and at intervals of 10 minutes blood sugars were determined.

The fraction of guinea pig immune serum which was precipitated at half saturation with ammonium sulfate was administered to mice as follows. The lyophilized material was dissolved in proper amount of buffered saline and injected into mice intravenously, intraperitoneally and subcutaneously. As shown in Fig. 2, the administration by any of three routes was followed by an elevation of blood sugar level.

These results suggested that endogenous insulin of rabbit and mouse was neutralized by guinea pig immune serum.

Guinea pigs, however, showed no change of blood sugar level by injection of guinea pig immune serum. This observation seemed that the endogenous guinea pig insulin as well as native insulin was not neutralized by their immune serum.

Skin Test—Skin test was carried out by injecting 0.1 ml. of inoculum containing 0.5 units of insulin intradermally to the back skin of the guinea pig. The sizes of erythema and induration appeared at the site of injection were observed afterwards.

Crystalline ox, whale and pig insulin were tested for skin reaction. In all cases, when immunized guinea pigs were tested as the recipients, the

erythema accompanying with induration at the site of injection became

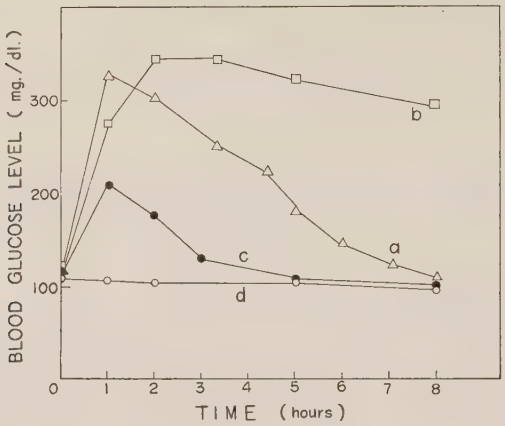

Fig. 2. Elevation of blood glucose level in mice following injection of 50 per cent $(NH_4)_2SO_4$-precipitated fraction of guinea pig immune serum. Fifty per cent $(NH_4)_2SO_4$-precipitated fraction of guinea pig immune serum was dissolved in buffered saline and injected into mice by three following routes. At interval of one hour, mice were sacrificed and blood sugar was determined.

a. intravenously : 0.5 ml. (4 u./ml.)
b. intraperitoneally : 1 ml. (2 u./ml.)
c. subcutaneously : 1 ml. (2 u./ml.)
d. intraperitoneally : 1 ml. of normal serum.

Table II

Skin Reaction of Guinea Pig Immunized

Dose of insulin *unit*	Diameter in *mm.*					
	After 3 hours			After 24 hours		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
2.0	22×23	25×30	26×28	20×21	18×20	15×20
1.0	24×20	25×24	30×28	20×20	19×22	21×18
0.5	21×20	28×26	25×28	15×13	18×15	12×15
0.25	18×15	20×18	20×21	10×11	16×12	12×12
0.1	8×10	12×15	11× 8	7× 5	13×10	8× 7
0.05	3× 5	6× 7	5× 4	2× 2	4× 4	3× 2
0.01	0	2× 3	2× 2	0	0	1× 2
Saline	0	0	0	0	0	0

One tenth ml. of saline containing the varying amounts of insulin was injected intradermally to guinea pig immunized (two weeks after three injections of antigen at interval of four weeks). The size of erythema was measured 3 and 24 hours after injection.

enlarged which reached maximum from two to three hours afterwards.

This reaction remained for 24 hours after injection and gradually weakened afterwards.

When varying amounts of insulin (2–0.1 units) were injected, the typical data using whale insulin are shown in Table II, demonstrating that the minimum dose of skin reaction was 0.1 units of insulin.

No change was demonstrable in normal guinea pig by injecting even with 2 units of insulin from any three species, while hypoglycaemic shock often occured.

Experimental Diabetes in Mice Induced by Guinea Pig Immune Serum—For this experiment, mice were chosen since their endogenous insulin were neutralized by guinea pig immune serum as previously described.

Six mice were injected by guinea pig immune serum and as a control six mice received similiar injections with normal guinea pig serum. Two peritoneal injections of 0.2 ml. and one subcutaneous injection of 0.3 ml. were administered to mice 3 times daily for three days.

Immune serum used here had activity of neutralizing 25 units insulin per ml. by mouse convulsion test.

The immune serum injected group became more irritable and took far more food and drank the water than the control group did. Nevertheless, the former group showed a marked decrease in body weight as shown in Fig. 3. Urine of each group was collected together on the 2nd and 3rd

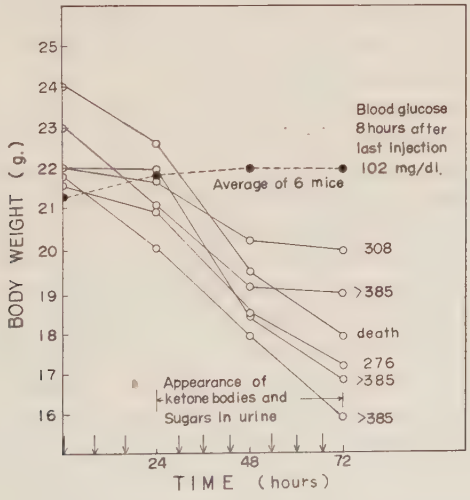

Fig. 3. Experimental diabetes induced in mice by injection of guinea pig immune serum. Six mice were injected by guinea pig immune serum 3 times daily for three days (at arrow mark). Mouse : ddN strain Immune serum ; Neutralizing activity 25 u./ml.

day and it was found that the mice of immune serum injected group excreted about four times of the volume of the urine of control group. The test for the presence of acetone bodies and sugars in urine was positive

in the former but negative in the latter each day. A remarkably hyperglycaemia was demonstrated by the determination of blood sugar level 8 hours after the last injection of immune serum, suggesting that considerable duration of high blood sugar level was followed by injection of immune serum.

These results were summarized in Fig. 3.

Diabetic syndrome, however, was transient: when the injection of immune serum was stopped or insulin was administered (0.2 units), the mice recovered from the symptoms above descrived. The pathological changes of pancreas of diabetes-induced mouse were not observed.

DISCUSSION

As it is well known, there are only slight differences among the amino acid sequences of various insulin preparations from the different animals, ox, pig, whale, sheep and horse.

In the present paper, the immunochemical similarity of insulin from several species as well as chemically structural similarity was demonstrated, using, insulin neutralizing technique and cutaneous reaction.

Neutralization of insulin by guinea pig immune serum against insulin was observed for even the bonito insulin (fish insulin), but not for guinea pig insulin. This fact may suggest that guinea pig insulin has the peculiar structure as compared to those from other species. Recently, Goldsmith and Moloney (*7*) demonstrated the difference of chromatographic behavior between guinea pig and ox insulin.

It is interesting that the endogenous insulin of mice and rabbits are also neutralized by guinea pig immune serum. Moloney and Coval (*5*), basing on the same observation, could produce a diabetic syndrome in mice by the injections of immune serum.

In the present investigation, experimental diabetes induced by the immunochemical method was confirmed to be reproducible.

Many methods have been attempted to produce experimental diabetes, but most of them were often drastic and difficult technically. On the contrary, the immunological method can produce insulin deficiency in animal more briefly and rapidly. In addition, insulin deficiency occurs as a result of neutralization basing on specific antigen-antibody reaction. The syndrome developed, therefore, is not complicated by any other changes. This will be recommened as a convenient and advantageous method of production of experimental diabetes.

The experimental diabetes in rabbit is attempted on the same standpoint by Wright (*10*).

SUMMARY

1. Guinea pig immune serum against ox insulin neutralized ox, pig, whale, dog, rabbit and bonite insulin but not guinea pig insulin.

2. Neutralization occurred also for mouse and rabbit endogenous insulin. Diabetic syndrome due to insulin deficiency was induced in mice by injections of guinea pig immune serum.

3. Immunized guinea pigs showed skin reaction for insulin from ox, pig and whale.

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THE CHEMISTRY OF LIPID OF POSTHEMOLYTIC RESIDUE OR STROMA OF ERYTHROCYTES

IX. SILICIC ACID CHROMATOGRAPHY OF MAMMALIAN STROMA GLYCOLIPIDS

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In 1951, Yamakawa and Suzuki isolated a ganglioside-like lipid, named hematoside, from equine red blood cells stroma (*1*). It was later confirmed by Klenk and Wolter (*2*) and further reexamined by Klenk and Lauenstein (*3*). Hematoside is now assumed to consist of lignoceric acid, sphingosine, two moles of hexose (mainly galactose besides a small amount of glucose) and N-glycollyl neuraminic acid (*4*). Klenk and Lauenstein were the first, however, to obtain a glycolipid from human erythrocytes (*5*). Further investigations by Yamakawa and Suzuki (*6*) as well as by Klenk and Lauenstein (*7*) have shown that the main glycolipid (named globoside) has an approximate structure of lignoceryl (or nervonyl) sphingosine acetyl-galactosamine tri- or tetra-hexoside. Yamakawa and Iida (*8*) found that globoside carries the capacity to inhibit the agglutination of erythrocytes of a given blood group by its corresponding isoagglutinin. From studies on the bovine erythrocytes by Klenk and Lauenstein (*7*), the hexosamine constituent is glucosamine, while Matsumoto (*9*) isolated from hog red cells a glycolipid, whose hexosamine is exclusively galactosamine. As has been emphasized by Yamakawa and co-workers (*10*), the glucosamine/galactosamine ratio of the lipid fraction of erythrocytes is quite characteristic of animal species.

On the other hand, Papirmeister and Mallette (*11*), while purifying the Forssman hapten from sheep red cells, noted that the essential substance for the activity was a glycolipid similar to globoside. Meanwhile, Kabat (*12*) doubted the group activity exhibited by globoside might be ascribed to a possible contamination of group mucoid. In contradiction to this, Yamakawa *et al.* (*13*) pointed out that the activity of globoside was much more stable toward dilute acid than mucoid group substance obtained from gastric mucin. More recently, Radin (*14*) attempted to fractionate globoside by cellulose column chromatography and reported that the ABO-activity was separated from main glycolipid peak. Shortly afterward, Yamakawa *et al.* (*15*) reported a similar finding using silicic acid column

and indicated the active portion was further divided into two fractions.

The present study is primarily concerned with the application of silicic acid chromatography to these and other several mammalian erythrocytes glycolipids. As the results, closely related glycolipids have been separated on the column by means of fractional elution with chloroform and methanol and the patterns obtained are remarkably characteristic and highly reproducible.

EXPERIMENTAL

Preparation of the Column—Columns of different sizes were prepared from 2 parts of silicic acid (Mallinckrodt, AR., suitable for chromatographic analysis, without activation) and one part of thoroughly washed dry Hyflo Super-Cel (Johns Mannville). In a typical experiment, 60 g. (40 g. + 20 g.) of mixed powder was suspended in chloroform to a rather thin slurry which was then poured into a chromatographic tube of 2.2 cm. diameter and allowed to settle.

Preparation of the Sample—Erythrocytes of various species of mammals were hemolyzed with 10 volumes of 0.3 per cent acetic acid solution, stroma precipitated was spun down, washed and freeze-dried. Extraction of this dry stroma with methanol-ether (1 : 1) mixture was repeated four times, and from this extract was obtained so-called M.E.-glycolipid by treatment with anhydrous ether and pyridine. M.E.-glycolipid was contained in the pyridine-soluble portion of ether-insoluble white matter separated from the extract. Methanol-ether extracted residual stroma gave so-called C.M.-glycolipid upon continuous extraction with chloroform-methanol (1 : 1); the dark brown extract thus obtained was concentrated *in vacuo*, dissolved in a small volume of chloroform and precipitated with acetone. The yield of total glycolipid amounted to about 2 per cent of dry stroma, and was variable depending on the procedure and animal species.

Addition of the Sample to the Column—About 1.2 g. of the glycolipid sample dissolved in minimal amount of chloroform was applied on top of the column and allowed to drain in slowly. The inner walls of the tube were then washed with a small amount of chloroform.

Elution of the Material—The elution of the material from the column could be accomplished by the use of chloroform-methanol mixture of stepwise increasing concentration of the latter. In a typical experiment with a column composed of 40 g. of silicic acid and 20 g. of Hyflo Super-Cel, the elution was carried out by successive application of each 200 ml. portion of chloroform, then 20, 40, 60 and 80 per cent methanol-containing chloroform and finally pure methanol.

Analysis of the Fractions—In all case, a quantitative determination of carbohydrate was performed on each fraction. A suitable aliquot of fraction was pipetted into a graduated test tube and after the solvent was evaporated to dryness by immersing in boiling water-bath, the residue was dissolved in 0.5 ml. of water. Five ml. of anthrone reagent (100 mg. of anthrone and 2 mg. of thiourea dissolved in 200 ml. of 66 per cent sulfuric acid) was added and after thorough mixing, the mixture was heated at 100° for 15 min. Optical density was measured in a Coleman 6A electro-photometer using D-galactose as standard. In some cases, sialic acid was determined as described previously (*16*). To 1 ml. of aqueous solution was added 1 ml. of Bial's oricinol reagent and heated at 100° for 22 minutes. As reference standard, N-acetyl neuraminic acid from human erythrocytes (*4*) was used in this determination.

In case of human stroma, ABO-group activity was assayed with each fraction by

isoagglutination inhibition technique and in case of sheep and cat blood cells, Forssman activity determination was carried out by sheep cell hemolysis inhibition.

Isolation and Analysis of Purified Glycolipid—Fractions corresponding to glycolipid peak on chromatogram were pooled. After the solvent was evaporated *in vacuo* nearly to dryness at 40°, the residue was redissolved in minimal amount of chloroform, precipitated by acetone, dried in a desiccator, weighed and analyzed.

Phosphorus (*17*), hexose, reducing value (as galactose) (*18*), hexosamine (as glucosamine HCl) (*19*) and sialic acid (*16*) were measured with this material. Glucosamine/galactosamine ratio was determined according to Gardell (*20*). For optical rotation measurement, Hitachi spectrophotometer (EPU-2A) was used.

RESULTS AND DISCUSSION

In general, M.E.-glycolipid contains rather more ceramide-oligohexoside, which is usually more readily soluble in chloroform. On the other hand, to C.M.-glycolipid belong such glycolipids as are hardly soluble in pure chloroform unless a small amount of methanol is added. It can be said that M.E.-glycolipids are rather faster eluted from silicic acid column than C.M.-glycolipids.

Moreover, evidences have been accumulated, that phospholipid, especially lecithin and sphingomyelin were completely extracted with methanol-ether. However, an additional amount of cephalin-type phospholipid was contained in C.M.-glycolipid portion, and on chromatography, the slower eluted mucolipid fractions were often observed to be contaminated by this phospholipid. The removal of this non-choline-containing phospholipid could be accomplished by repeated chromatography on silicic acid, but not by Florisil.

Note on Nomenclature—The name, ceramide-oligohexoside*, is given to the class of glycolipids containing neither hexosamine nor sialic acid. The authors use the term, mucolipid, for such glycosphingoside as possessing hexosamine and/or sialic acid.

Fractionation of Human Erythrocytes Glycolipids (Globoside)—Crude globoside (*6*) was divided into several fractions (Fig. 1.). Fr. II were considered to contain ceramide-trihexosides. Fr. IV was highest in yield and composed of ceramide, three hexose and acetyl-galactosamine. Blood group activity appeared thereafter in Fr. VI and VII. In these more slowly eluted lipids, glucosamine and sialic acid were also present besides galactosamine. The detailed immunochemical study of these group-active fractions will be reported shortly after elsewhere.

Fr. II isolated by silicic acid chromatography of mixed lipids revealed the presence of a significant amount of phosphorus. That this phosphorus was indeed a contaminant and not a constituent of glycolipid was illustrated by the fact that it could be removed by rechromatography on silicic acid and on Florisil column according to the procedure of Radin (*21*). Fr. II

* Rapport, M. M., and Alonzo, N. (*Federation Proc.*, **18**, 307 (1959)) have proposed the name 'cytoside' for tumor-specific glycolipid of similar structure. But it seems not yet to be determined whether cytoside is really ceramide-oligohexoside itself.

of M.E.- as well as C.M.-glycolipid were collected and rechromatographed on silicic acid column by eluting with solvent mixtures of chloroform-methanol 9 : 1, 8.5 : 1.5, and 8 : 2. The low-phosphorus (P 0.38) lipid (550 mg.) thus obtained was dissolved in a small amount of chloroform-methanol (2 : 1), passed through a column of Florisil (25 g.) and eluted with 300 ml. of this solvent mixture. Thus, an apparently pure phosphorus-free glycolipid was obtained.

Analysis: C 63.4, H 10.2, N 1.36, Hexose 48.4, Reducing value, 49.2, Hexosamine, 0.35, $[\alpha]_{589}$ −9.7° (10 per cent in pyridine).

On the basis of quantitative determination, it appears the material is most probably lignoceryl sphingosine trigalactoside.

Calculated for $C_{60}H_{113}NO_{18}$ (1135)
C 63.4 H 10.0 N 1.23 Hexose 47.6

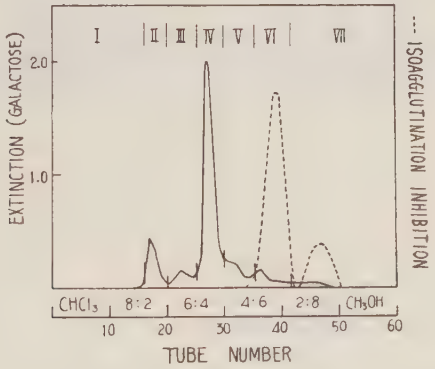

Fig. 1. Silicic acid chromatography of human erythrocytes M.E.-glycolipid.

Table I

Composition of a Typical Case of Human Erythrocytes M.E.-Glycolipid
(Corresponding to Fig. 1.)

	Original Mixture	Fraction No.						
		I	II	III	IV	V	VI	VII
Weight, mg	1200	14	200	48	449	55	30	152
Hexose, %	28.9	3.30	24.8	38.2	45.3	39.3	25.6	2.43
Hexosamine, %	—	0	0	1.00	13.3	10.7	11.2	2.20
Phosphorus, %	1.52	—	2.20	—	—	—	—	—

One g. of Fr. IV (Fig. 1) or Fr. III (Fig. 2) was loaded on a column composed of 50 g. of Florisil. Each 310 ml. of solvent mixtures of chloro-

form-methanol, 2:1 and 1:1, was sufficient to allow separation of essentially phosphorus-free mucolipid. 538 mg. of this mucolipids was further purified on silicic acid column (60 g. of mixed powder) by eluting with 600 ml. of chloroform-methanol (7:3). An apparently single glycolipid peak was obtained.

Analysis:

Found: C 59.23–60.95, H 9.49–10.23, N 2.23, Hexose 49.5–50.0, Hexosamine 17.4–17.5, Reducing val. 62.4–67.5

Sialic acid was not detectable. $[\alpha]_{589}=+19.5°$ (10% in pyridine)

Calculated for lignoceryl sphingosine acetylgalactosamine trihexoside $C_{68}H_{126}N_2O_{23}$: C 60.96, H 9.48, N 2.09, Hexose 53.8, Hexosamine 16.1

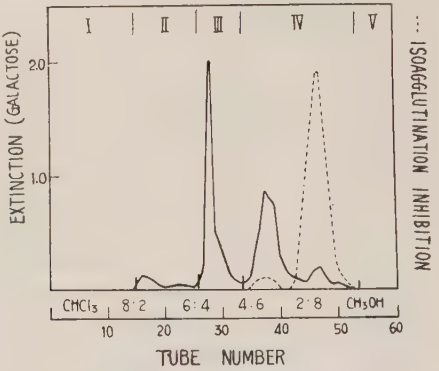

Fig. 2. Silicic acid chromatography of human erythrocytes C.M.-glycolipid.

Table II

Composition of a Typical Case of Human Erythrocytes C.M.-Glycolipid (corresponding to Fig. 2.)

	Original Mixture	Fraction No.				
		I	II	III	IV	V
Weight, mg	980	—	188	**125**	179	73
Hexose, %	14.6	—	5.12	**35.2**	23.7	3.41
Hexosamine, %	—	—	0.47	**11.6**	10.0	1.43
Phosphorus, %	3.00	—	3.26	**1.07**	1.96	—

By Gardell's procedure, the existence of only galactosamine as amino sugar was established. The results of the analysis corresponds to lignoceryl sphingosine acetylgalactosamine trihexoside previously assumed for globoside (*6*). The value of optical rotation formerly reported, +10.3°, was too low,

possibly since the material was mixed with levo-rotatory ceramide oligohexoside. On hydrolysis, galactose and glucose were detected by paper chromatography. This purified main glycolipid gave clear aqueous solution, and when heated it turned turbid and became clear again by followed cooling. Contrary to the previous finding (*22*), this aqueous solution was not viscous and gave apparently single boundary and sedimentation coefficient comparable with those of brain ganglioside (*22*), strandin (*23*, *24*) and brain mucolipid (*25*) in the ultracentrifugal field (Fig. 3, 4).

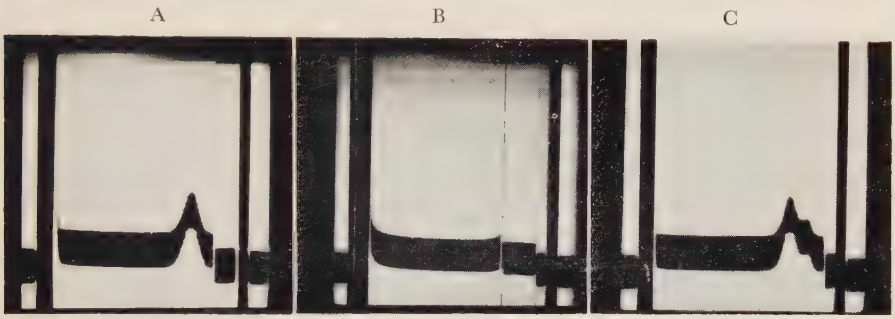

Fig. 3. Sedimentation patterns of purified mucolipids.
A. Globoside (purified Fr. IV., Fig. 1), 0.4% in 0.85% NaCl
35,600 r.p.m., $s_{..,w} = 10.9$ S
B. Hematoside, the latter peak (Fr. VI, Fig. 5), 0.4% in water
35,600 r.p.m., $s_{20,w} = 24.4$ S
C. Hematoside, the former peak (Fr. IV, Fig. 5), 0.4% in water
35,610 r.p.m.

Blood group active portions were concentrated in Frs. VI and VII in case of M.E.-, and in Fr. IV in case of C.M.-glycolipid. In both cases, rechromatography on silicic acid divided them into two distinctly separated group-active fractions and the analytical values of these materials, although not yet pure, are as follows.

Active portion *I* Hexose 33.2, hexosamine 13.2, sialic acid 5.3
,, ,, *II* ,, 40.7, ,, 14.5, ,, ,, 2.1
Glucosamine/galactosamine ratio of active portion *I* is 1.0 and *II* is 1.5.

Fractionation of Equine Erythrocytes Glycolipids (Hematoside)—In this case, the yield of M.E.-glycolipid was found to be extremely small and the mucolipids (hematosides) were present abundantly in C.M.-glycolipid. This tendency was already observed as reported previously (*1*). Data from a typical experiment are presented in Figs. 4 and 5 together with Tables III and IV.

The relatively high content of phosphorus in the fast running peak (Fr. II) made it seem desirable to remove phospholipid further by usual Florisil technique. However, in this case, the trial was accomplished only with difficulty. Moreover, the clean-cut separation of hematosides fractions (Frs. IV, V and VI of Fig. 5) was not successful by rechromatography of each fraction, but the individual existency of Frs. IV and VI were repeat-

edly indicated. Because of the similarity of composition, a postulation that Fr. VI might be a salt form of Fr. IV is indeed possible, though several attempts with or without acidification as well as electrodialysis prior to

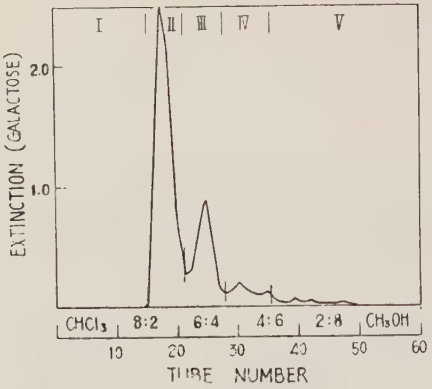

Fig. 4. Silicic acid chromatography of equine erythrocytes M.E.-glycolipid.

TABLE III

Composition of a Typical Case of Equine Erythrocytes M.E.-Glycolipid (corresponding to Fig. 4.)

	Original Mixture	Fraction No. I	II	III	IV	V
Weight, mg	600	—	356	19	65	8
Hexose, %	6.26	—	8.72	18.5	2.74	2.91
Hexosamine, %	—	—	0.13	0.40	0.31	0.26
Sialic acid, %	—	—	—	10.3	0.73	0
Phosphorus, %	3.09	—	—	1.56	2.98	—

chromatography did not yet give concrete evidence for this assumption. The phosphorus-free specimen of Frs. IV and VI showed similar optical activity, $[\alpha]_{589} = -14.5°$ (1.86 per cent in pyridine) and $-17.2°$ (1.16 per cent), respectively, but aqueous solution of the latter gave similar extraordinary boundary (Fig. 3, B) in the ultracentrifugal field as reported already with blood cells mucolipids (*22*), whereas the sedimentation pattern of purified Fr. IV represented apparently two different components (Fig. 3, C).

In several experiments, Frs. VII as well as VIII contained considerable amount of sialic acid and hexosamine, but in another instance only sialic acid was present. The presence of such a hexosamine-containing

glycolipid in equine stroma was already described by Klenk and Lauenstein (*3*).

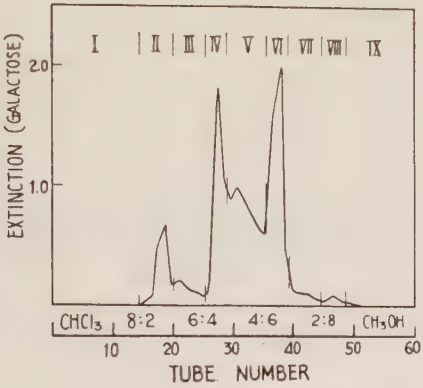

Fig. 5. Silicic acid chromatography of equine erythrocytes C.M.-glycolipid.

Table IV

Composition of a Typical Case of Equine Erythrocytes C.M.-Glycolipid
(corresponding to Fig. 5.)

	Original Mixture	Fraction No.								
		I	II	III	IV	V	VI	VII	VIII	IX
Weight, mg	900	—	275	18	**131**	185	**200**	22	16	6
Hexose, %	16.0	—	5.25	1.67	**28.0**	34.5	**35.9**	27.5	15.4	—
Hexosamine, %	1.10	—	0.44	—	**0.71**	0.44	**0.71**	4.51	3.22	—
Sialic acid, %	20.7	—	1.25	0.68	**19.8**	26.7	**29.3**	22.5	58.7	—
Phosphorus, %	1.34	—	3.36	—	**0.47**	0	**0.08**	0.96	—	—

Fractionation of Bovine Erythrocytes Glycolipids—As illustrated in Figs. 6 and 7, the M.E.-glycolipid contains nearly whole amount of ceramide-oligohexoside and only a small portion of glucosamine-containing mucolipid, while in the C.M.-glycolipid, the former is almost negligible and the latter larger in amount. In this fraction (Fr. IV) the existence of sialic acid was also indicated, however in another experiment, this mucolipid possessed hexosamine but no sialic acid. This strange finding was also already described by Klenk and Lauenstein (*7*).

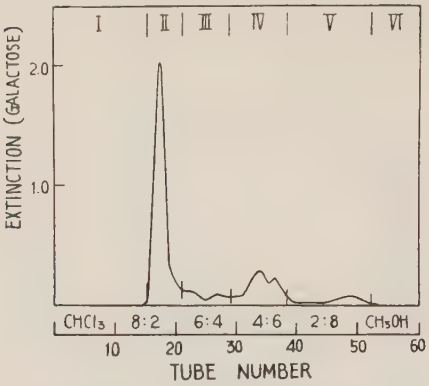

Fig. 6. Silicic acid chromatography of bovine erythrocytes M.E.-glycolipid.

Table V

Composition of a Typical Case of Bovine Erythrocytes M.E.-Glycolipid (corresponding to Fig. 6.)

	Original Mixture	Fraction No.					
		I	II	III	IV	V	VI
Weight, mg	530	—	136	30	**16**	187	18
Hexose, %	6.71	—	14.2	10.5	**34.3**	1.86	2.76
Hexosamine, %	—	—	0.14	1.66	**7.97**	0.48	—
Sialic acid %	—	—	—	—	**2.73**	0	—
Phosphorus, %	3.30	—	2.92	—	**0.96**	3.77	—

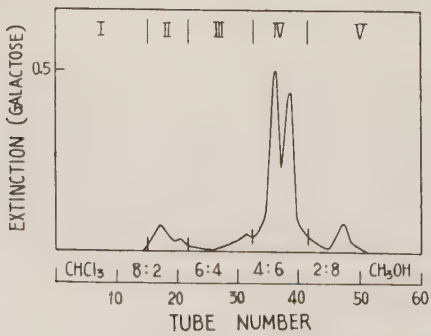

Fig. 7. Silicic acid chromatography of bovine erythrocytes C.M.-glycolipid.

Table VI

Composition of a Typical Case of Bovine Erythrocytes C.M.-Glycolipid (corresponding to Fig. 7.)

	Original Mixture	Fraction No.				
		I	II	III	IV[a)]	V
Weight, mg	850	—	533	84	**61**	9
Hexose, %	4.55	—	0.82	2.02	**25.8**	1.51
Hexosamine, %	—	—	0.02	0.32	**6.59**	4.15
Sialic acid, %	—	—	—	0	**10.8**	—
Phosphorus, %	3.44	—	3.87	—	**1.56**	—

a) In another experiment: Hexose 24.6, Hexosamine 8.70, Sialic acid 0.76.

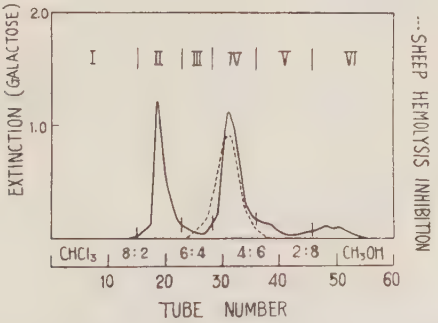

Fig. 8. Silicic acid chromatography of sheep erythrocytes M.E.-glycolipid.

Table VII

Composition of a Typical Case of Sheep Erythrocytes M.E.-Glicolipid (corresponding to Fig. 8.)

	Original Mixture	Fraction No.					
		I	II	III	IV	V	VI
Weight, mg	504	—	180	10	**54**	46	100
Hexose, %	9.24	—	7.36	11.2	**23.3**	6.05	0.97
Hexosamine, %	—	—	0.21	—	**14.4**	2.64	—
Sialic acid %	—	—	0	0	**0**	0	—
Phosphorus, %	2.80	—	2.64	2.48	**0.36**	2.66	3.18

Fractionation of Sheep Erythrocytes Glycolipids—Sheep red cells are well known carrier of Forssman antigen. Forssman activity existed in M.E.-glycolipid and on silicic acid column, the active fraction coincided in position with the main mucolipid, which contained galactosamine and glucosamine at a ratio of 4:1 but no sialic acid. In C.M.-glycolipid there was no Forssman activity, indicating the active material is more or less loosely bound with stroma.

The main mucolipid (Fr. IV in Fig. 8) inhibited sheep cell hemolysis in amount as little as 1 μg. and after purification, showed the following composition.

Hexose 36.9, hexosamine 17.4, reducing value 56.6, N 2.37, P 0.15

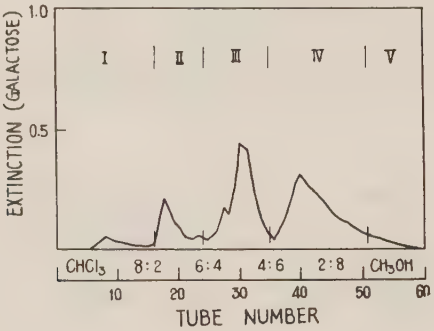

Fig. 9. Silicic acid chromatography of sheep erythrocytes C.M.-glycolipid.

Table VIII

Composition of Sheep Erythrocytes C.M.-Glycolipid
(corresponding to Fig. 9.)

	Original Mixture	Fraction No.				
		I	II	III	IV	V
Weight, mg	600	—	243	48	51	1
Hexose, %	4.00	—	1.72	6.30	5.64	—
Hexosamine, %	—	—	0.32	2.46	3.69	—
Sialic acid, %	—	—	0	0	0	—
Phosphorus, %	3.28	—	3.07	1.75	0.43	—

The structural relationship between these blood cells mucolipids and the Forssman hapten obtained from equine spleen and kidney (*26*) will be discussed elsewhere*.

* Yamakawa, T., and Makita, A., unpublished.

Fractionation of Cat Erythrocytes Glycolipid—It is said that cat red cells have also Forssman antigen. Similarly as in the case of sheep cells, the activity was found only in M.E.-glycolipid, but in far less degree. In Figs. 10 and 11, the later peak composed the main mucolipid, which, resemblance to hematoside, contained much sialic acid and a negligible amount of hexosamine. Forssman active portion was eluted before this main mucolipid, corresponding to the active portion in case of sheep red cells (Fig. 8). The data on the composition of these fractions are tabulated in Tables IX and X.

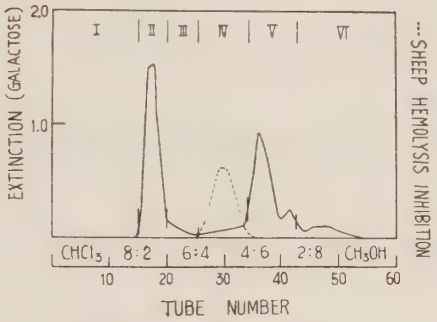

Fig. 10. Silicic acid chromatography of cat erythrocytes M.E.-glycolipid.

Table IX

Composition of a Typical Case of Cat Erythrocytes M.E.-Glycolipid
(corresponding to Fig. 10.)

	Original Mixture	Fraction No.					
		I	II	III	IV	V	VI
Weight, mg	300	—	88	39	19	**37**	120
Hexose, %	9.05	—	10.5	1.45	10.1	**23.6**	2.67
Hexosamine, %	—	—	0.23	0.36	2.16	**2.04**	0.43
Sialic acid, %	2.79	—	0.88	0.50	3.33	**28.3**	2.26
Phosphorus, %	3.08	—	2.04	—	—	**1.44**	3.69

After rechromatography on silicic acid and recrystallization from methanol, the main mucolipid (Fr. IV of Fig. 11) gave following analytical value.

C 55.58, H 8.07, N 2.62, Hexose 23.4–24.4, Hexosamine 0.11, P 0.17
Sialic acid 53.0, $[\alpha]_{589} = -20.1°$ (3.78 per cent in pyridine)

From these data, it might be assumed that this mucolipid possibly

consist of ceramide, three moles of hexose and three or four moles of sialic acids.

Calcd. for ceramide trihexoside plus 3 sialic acids, $C_{93}H_{164}N_4O_{42}$,
C 55.6 H 8.17 N 2.79 Hexose 26.9 Sialic acid 46.1
Calcd. for ceramide trihexoside plus 4 sialic acids, $C_{104}H_{181}N_5O_{50}$,
C 54.5 H 7.86 N 3.05 Hexose 23.5 Sialic acid 53.5

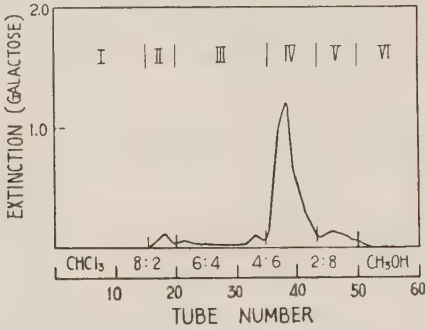

Fig. 11. Silicic acid chromatography of cat erythrocytes C.M.-glycolipid.

Table X

Composition of a Typical Case of Cat Erythrocytes C.M.-Glycolipid
(corresponding to Fig. 11.)

	Original Mixture	Fraction No.					
		I	II	III	IV	V	VI
Weight, mg	450	—	27	58	**275**	44	26
Hexose, %	23.2	—	7.99	8.69	**27.8**	13.9	8.53
Hexosamine, %	—	—	—	0.75	**0.76**	2.08	1.45
Sialic acid, %	38.5	—	2.93	6.03	**37.2**	47.0	18.0
Phosphorus, %	1.32	—	1.62	1.42	**0.42**	1.50	—

Fractionation of Guinea-Pig Erythrocytes Glycolipids—In the M.E.-glycolipid (Fig. 12), main mucolipid was eluted so fast that it could be hardly separable from ceramide-oligohexoside. Rechromatography of this fraction (Fr. III) with Florisil column and infra-red spectrum indicated the presence of ceramide-oligohexoside but a complete separation in a single peak was not accomplished. Analysis showed it as a mucolipid containing only galactosamine as hexosamine component and no sialic acid. In this respect, it resembles to main globoside of human erythrocytes. However, the direction of optical rotation was found quite opposite.

Analysis for purified mucolipid from Fr. III,

Hexose 47.1, hexosamine 15.7, reducing val., 45.5,
N 2.08, P 0.08, $[\alpha]_{589} = -14.6°$ (2.67 per cent in pyridine)

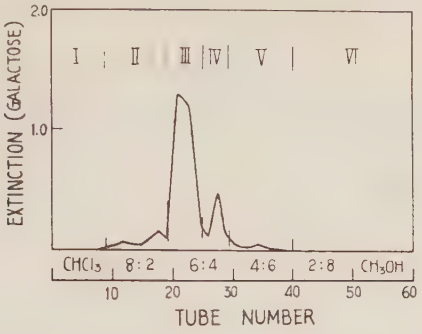

Fig. 12. Silicic acid chromatography of guinea-pig erythrocytes M.E.-glycolipid.

Table XI

Composition of Guinea-Pig Erythrocytes M.E.-Glycolipid

	Original Mixture	Fraction No.					
		I	II	III	IV	V	VI
Weight, mg	800	—	170	**402**	**77**	30	2
Hexose, %	25.3	—	5.00	**36.9**	**23.4**	8.95	—
Hexosamine, %	—	—	0.42	**8.10**	**6.97**	1.89	—
Sialic acid, %	—	—	0	**0**	**0**	0	—
Phosphorus, %	1.12	—	2.14	**0.24**	**0.13**	—	—

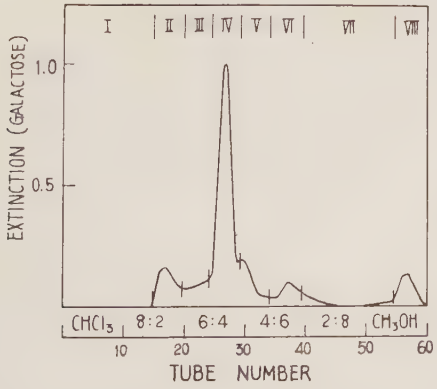

Fig. 13. Silicic acid chromatography of guinea-pig erythrocytes C.M.-glycolipid.

C.M.-glycolipid of guinea-pig erythrocytes consisted mainly of mucolipid (Fr. IV, Fig. 13), which appeared to be the same as described above for M.E.-glycolipid, but the amount of the material was so small that no detailed examination could be carried out.

TABLE XII

Composition of Guinea-Pig Erythrocytes C.M.-Glycolipid

	Original Mixture	Fraction No.							
		I	II	III	IV	V	VI	VII	VIII
Weight, mg	160	—	26	3	**19**	3	4	1	17
Hexose, %	8.53	—	6.55	95.9	**25.8**	1.92	9.16	—	4.58
Hexosamine, %	—	—	—	5.73	**10.9**	—	—	—	—
Sialic acid, %	—	—	—	0	**0**	—	—	—	—

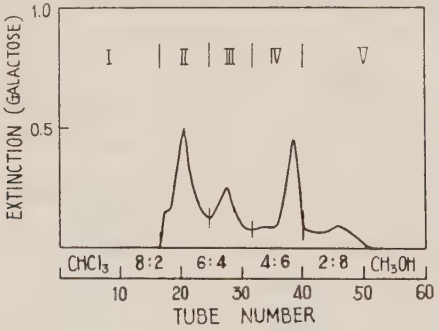

FIG. 14. Silicic acid chromatography of rabbit erythrocytes M.E.-glycolipid.

TABLE XIII

Composition of Rabbit Erythrocytes M.E.-Glycolipid
(corresponding to Fig. 14.)

	Original Mixture	Fraction No.				
		I	II	III	IV	V
Weight, mg	150	—	43	13	8	37
Hexose, %	7.47	—	8.91	18.0	24.5	1.72
Hexosamine, %	—	—	0.23	1.2	5.0	0.51
Sialic acid, %	—	—	0	0	0	0

Fractionation of Rabbit Erythrocytes Glycolipids—Since the yield of stroma

from rabbit erythrocytes was low, freeze-dried erythrocytes were used as the starting material. In this case, too, analysis was carried out only with partially purified specimen because of the small amounts of glycolipid obtained. However, the chromatographic pattern was remarkably peculiar and several glycolipids peaks were demonstrated. Because the starting material was erythrocyte itself, the fractions were heavily contaminated with considerable amounts of non-lipid, probably inorganic materials.

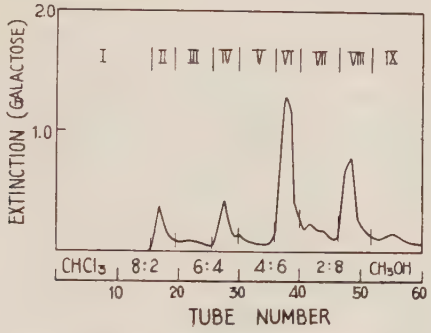

Fig. 15. Silicic acid chromatography of rabbit erythrocytes C.M.-glycolipid.

Table XIV

Composition of Rabbit Erythrocytes C.M.-Glycolipid (corresponding to Fig. 15.)

	Original Mixture	Fraction No.								
		I	II	III	IV	V	VI	VII	VIII	IX
Weight, mg	730	—	14	12	10	27	65	100	127	56
Hexose, %	2.41	—	—	—	14.7	1.38	12.9	2.15	3.42	2.84
Hexosamine, %	—	—	—	—	—	—	2.42	—	1.70	—

After partial purification by removal of contaminant inorganic materials, the main mucolipids from C.M.-glycolipid gave the following value.

Fr. VI Hexose 40.8, hexosamine 9.9, sialic acid 0.
Fr. VIII ,, 16.0, ,, 4.9, ,, ,, 0.

Analysis indicated that the mucolipids of rabbit erythrocytes contained hexosamine but no sialic acid.

Infra-Red Determination—During the chromatographic procedure, the process of purification was checked by infra-red spectrum. Purified glycolipid specimen gave no absorption band at 1750 cm^{-1}. assigned to ester C=0 stretching frequency. As illustrated in Fig. 16, the absorption patterns in region from 1650 cm^{-1}. to 1000 cm^{-1}. were characteristic of each glycolipid. The samples of cerebroside (I) and cerebroside sulfuric ester (II) were

prepared from hog brain and purified by means of silicic acid chromatography. The pattern of (I) is quite in accordance with the diagram reported by Chatagnon (*27*). Strong absorption found for (II) at near 1245 cm^{-1}. is considered to be that of sulfuric acid (*28*). In general, cerebroside or ceramide-oligohexoside shows sharp absorption band at 1475 cm^{-1}. as compared with mucolipids. Mucolipids from various erythrocytes gave essentially similar pictures; apparently no significant differences are observed whether the mucolipid contains hexosamine or sialic acid.

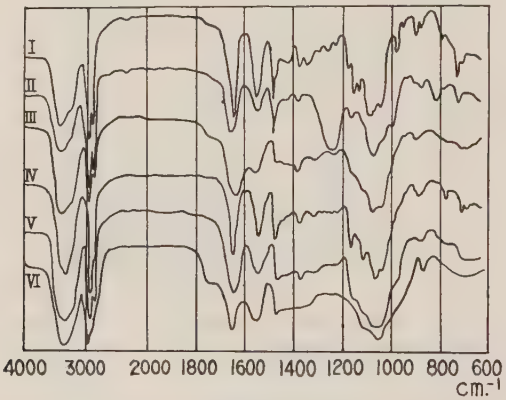

Fig. 16. Infra-red spectra of various glycolipids. (Hitachi EP-I 2, double-beam automatic recording spectophotometer, pressed in KBr) (I) Hog brain cerebroside, (II) Hog brain CSE, (III) Beef brain ganglioside, (IV) Ceramide oligohexoside of human erythrocytes, (V) Main mucolipid of human erythrocytes, (VI) Main mucolipid of cat erythrocytes.

SUMMARY

1. Silicic acid column chromatography was applied to the purification of crude glycolipids obtained from human, equine, bovine, sheep, cat, guinea-pig blood cells stroma as well as rabbit erythrocytes.

2. Glycolipids were separated to methanol-ether soluble and chloroform-methanol soluble materials and each of these was further divided by chroma- into ceramide-oligohexoside and mucolipid.

3. Chromatographic pattern and nature of mucolipids were remarkably peculiar to each animal secies. Generally speaking, muco lipids of human, sheep, guinea-pig and rabbit contain hexosamines, whereas those of equine and cat sialic acids instead.

4. Blood group active lipid as well as Forssman hapten were purified by this procedure from human, sheep and cat erythrocytes.

5. Infra-red spectra of these and other glycolipids are presented.

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STUDIES ON SULFATASES OF THE LIVER OF CHARONIA LAMPAS

II. PURIFICATION AND PROPERTIES OF SULFATASES

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An enzyme cellulosepolysulfatase, hydrolysing sulfuric ester bonds in cellulosepolysulfate was found in the liver extract of *Charonia lampas* (*1*). The enzyme preparation contains also a polysaccharase system including cellulase which attacks the polysaccharide moiety in cellulosepolysulfate.

The present report describes the methods of purification of arylsulfatase, glucosulfatase and cellulosepolysulfatase in the liver extract and the separation of them from the polysaccharase system. Some properties of these enzymes are presented too.

MATERIALS AND METHODS

Preparation of Enzyme Solution and Determination of Enzyme Activities—The methods used were described previously (*1*), (*2*). Enzyme unit is defined as enzyme quantity liberating 1 μmole of sulfate (glucose in the case of polysaccharases) per hour under optimum conditions. Specific activity is defined as number of unit per mg. protein.

Preparation of Substrates—The potassium salts of nitrocatechol sulfate (NCS), glucose 6-monosulfate (GS) and cellulosepolysulfate were prepared by the methods described previously (*1*) (*2*).

Carboxymethyl-cellulose (CM-cellulose)—Prepared by the method of Peterson and Sober (*3*), and the method, as described by them for the separation of proteins by CM-cellulose column chromatography, was also followed. Effluent fractions were assayed for sulfatases and protein.

N,N-Diethylaminoethyl-cellulose (DEAE-cellulose)—Commercial (Eastman Organic Chemicals) preparations were used.

RESULTS

A. Purification of Sulfatases—With 100 ml. of crude extract of the liver of *Charonia lampas* as starting material, a preliminary concentration of the sulfatases (arylsulfatase, glucosulfatase and cellulosepolysulfatase) was carried out by the treatment with trypaflavin as previously described (*1*). Polysaccharases precipitated in a great amount from the crude extract by adding trypaflavin, while sulfatases were not precipitated. Enzyme solution prepared in this way ("preparation A"), contained less than 10 per cent of

Table I

Stages in the Purification of the Sulfatases of the Liver of Charonia lampas

Enzyme unit is defined as enzyme quantity liberating 1 μmole of sulfate (glucose in the case of polysaccharases) per hour under optimum conditions.

Specific activity is defined as number of unit per mg. protein.

Enzymes \ Procedures		Crude extract	Trypaflavin ("preparation A")	Paper electrophoresis	CM-cellulose ("preparation B")
Arylsulfatase					
Specific activity		4.55	15.5	43.6	78.3
Total activity	units	2900	1860	840	1102
remaining	(%)	100	64	29	38
Glucosulfatase					
Specific activity		0.32	3.06	3.4	50
Total activity	units	202	200	121	46.5
remaining	(%)	100	99	60	23
Cellulosepolysulfatase					
Specific activity		0.20	0.29	0.37	1.20
Total activity	units	128	38.4	25.6	37.0
remaining	(%)	100	30	20	29
Polysaccharases					
Specific activity		0.022	0.008	0.23	—
Total activity	units	94.6	6.8	28.2	—
remaining	(%)	100	7.3	30	—

original polysaccharase activity. Further attempts were tried to purify the sulfatases by electrophoresis and by adsorption with CM-cellulose, DEAE-cellulose or ion-exchange resin. Table I shows specific activity and total activity of enzymes obtained at various stages of purification.

1. Electrophoretic Fractionation of "Preparation A"—Three ml. solution (14 mg. protein) of "preparation A" was concentrated by freeze-drying to 0.1 ml. and run for 17 hours on horizontally held Toyo-roshi No. 50 filter paper, width 7.5 cm., at 200 volt./40 cm. in the presence of 0.02 *M* acetate buffer pH 3.5. The paper was dried at room temperature and cut into pieces of 1.5 cm. wide by 1 cm. long each. Protein was located by the method of Lowry *et al.* (*4*), sulfatase and polysaccharase activities were located according to the methods previously described (*1*), (*2*). The three sulfatases were more basic than polysaccharases, the latter, migrated towards the cathode at the much slower rate than the former. Sulfatases were thus separated from polysaccharases. The cellulosepolysulfatase preparation did not liberate reducing substances from cellulosepolysulfate (Fig. 1).

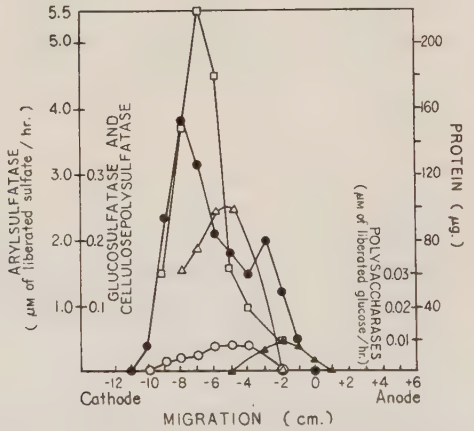

FIG. 1. Paper electrophoresis. 3 ml. solution (14 mg. protein) of "preparation A" was concentrated to 0.1 ml. by freeze-drying and run on Toyo-roshi No. 50 filter paper in 0.02 *M* acetate buffer pH 3.5 for 17 hours at 200 volt./40 cm. ---□--- arylsulfatase, ---△--- glucosulfatase, ---○--- cellulosepolysulfatase, ---▲--- polysaccharases and ---●--- protein.

Other attempts to fractionate "preparation A" by paper electrophoresis and starch electrophoresis at various pH, that is, pH 5.0, 6.5, 7.0 and 8.6 were carried out. In all cases of pH, sulfatases could be separated from polysaccharases. However three sulfatases could not be separated from each other. At pH 8.6, each sulfatase activity was lost to a considerable degree.

2. Adsorption with CM-cellulose—Approximately 3 g. of adsorbent was used to prepare packed column of 0.70 $cm^2 \times 30$ cm. Eight ml. solution (37

mg. protein) of "preparation A" in the presence of pH 5.2, 0.01 *M* acetate buffer, was passed through a CM-cellulose column bufferized with 0.01 *M* sodium acetate pH 5.2. By washing the column with about 100 ml. of 0.01 *M* sodium acetate pH 5.2, polysaccharase fraction was removed. The elution diagram obtained is shown in Fig. 2. Gradients of pH and salt concentration were employed for elution. All column operations were performed in a cold room. Considerable purification of sulfatases was achieved by the use of this technique. Especially specific activity of glucosulfatase in this preparation ("preparation B") was 50. This represents a 160-fold purification of glucosulfatase with a retention of 23 per cent of the original activity (Table I).

3. Adsorption with DEAE-cellulose—In Fig. 3, is shown the elution dtagram obtained when "preparation B" was chromatographed on the anion exchanger DEAE-cellulose. The starting buffer 0.01 *M* sodium acetate at pH 6.0 was continued to flow in order to remove polysaccharases, after which, gradients of pH and salt concentration were employed for elution. Fig. 3 was quite similar to Fig. 2. But activities of glucosulfatase and cellulose-

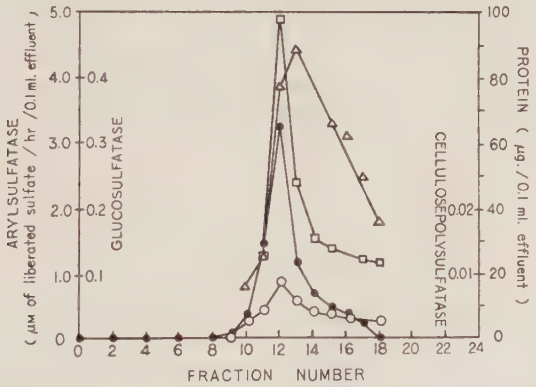

Fig. 2. Effuluent diagram of "preparation A" with CM-cellulose. 8 ml. solution (37 mg. protein) of "preparation A" applied to 3 g. of CM-cellulose; effuluent collected in 5 ml. fractions; flow rate, 2 ml./hour. Buffers: 0.01 *M* sodium acetate, pH 5.2 gradient to 0.5 *M* sodium acetate pH 6.0. Mixing chamber volume, 250 ml. ---□--- arylsulfatase, ---△--- glucosulfatase, ---○--- cellulosepolysulfatase and ---●--- protein.

polysulfatase were lost to a considerable degree.

Chromatography of "preparation A" on column of ion-exchange resin IRC-50 resulted in an elution diagram, which was also similar to that in Fig. 2.

As will be seen from the above experiments, it was not so difficult to separate sulfatases from polysaccharases, while three sulfatases could not be searated from each other since they behaved together and showed similar

properties throughout the process of these enzyme purifications. After all, treatment with trypaflavin of the crude liver extract was the most effective

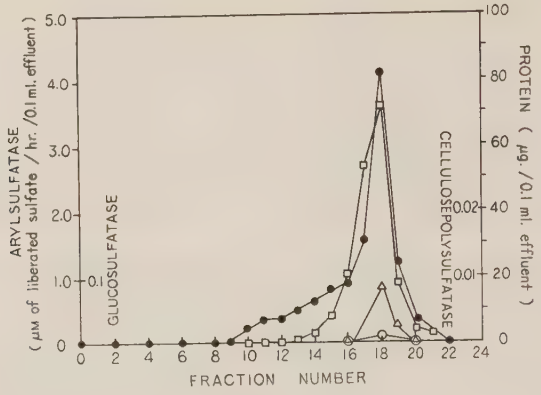

FIG. 3. Effluent diagram of "preparation B" with DEAE-cellulose. 5 ml. solution (4 mg. protein) of "preparation B" applied to 2 g. of DEAE-cellulose; effluent collected in 5 ml. fractions; flow rate 4 ml./hour. Buffers: 0.01 *M* sodium acetate, pH 6.0 gradient to 0.5 *M* sodium acetate pH 5.2. Mixing chamber volume, 200 ml. ---□--- arylsulfatase, ---△--- glucosulfatase, ---○--- cellulosepolysulfatase and ---●--- protein.

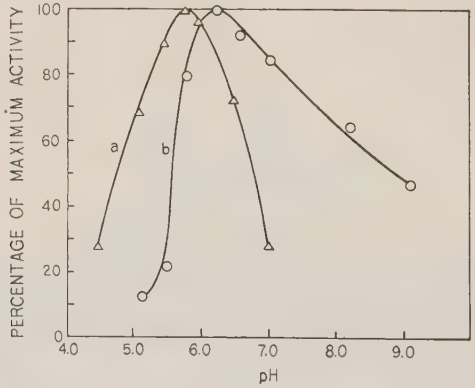

FIG. 4. pH-activity curve for gucosulfatase (a) and cellulosepolysulfatase (b). Enzyme activity was measured at 37° for 2 hours (a) and 3 hours (b) respectively, in the presence of 0.1 *M* acetate (pH 4.0–5.7), tris-maleate (pH 5.1–7.0) and tris-hydrochloride (pH 6.8–9.1) buffers adjusted to the required pH. (a) ---△--- glucosulfatase and (b) ---○--- cellulosepolysulfatase.

as preliminary purification technique for the three sulfatases. For arylsulfatase, adsorption with DEAE-cellulose of "preparation B" was the most

effective. Specific activity of the enzyme was 87. This represents a 20-fold purification of the enzyme with a retention of 28 per cent of original activity. For glucosulfatase and cellulosepolysulfatase, adsorption technique with CM-cellulose of "preparation A" was the most effective. Specific activity of glucosulfatase was 50. This represents a 160-fold purification of glucosulfatase with a retention of 23 per cent of the original activity. Specifiic activity of cellulosepolysulfatase in "preparation B" was 1.2. This represents a 6-fold purification of the enzyme with a retention of 29 per cent of the original activity.

B. Properties of Sulfatases in "Preparation B"—pH activity curve of cellulosepolysulfatase in "preparation B" was different from that obtained in the crude extract. Maximum enzyme activity was obtained at pH 5.1–5.5 in the experiment with crude extract and pH 6.2 in the case with "preparation B" (Fig. 4).

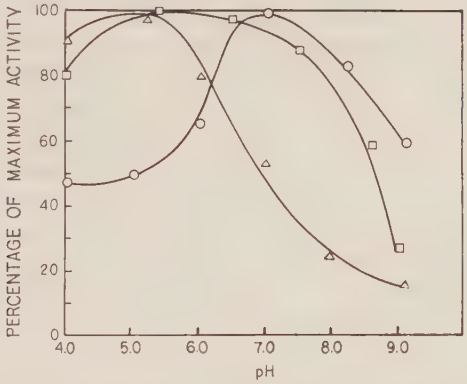

FIG. 5. Inactivation of sulfatases in "preparation B" at various pH. "Preparation B" was adjusted to the required pH with 0.02 M acetate and tris buffers, and allowed to stand at 4° for 24 hours. The pH of the mixtures were readjusted to 0.2 *M*, pH 6.0 and the activities of three sulfatases were determined. ---□--- arylsulfatase, ---△--- glucosulfatase and ---○--- cellulosepolysulfatase.

In Fig. 5, "preparation B" was adjusted to the required pH with 0.02 *M* acetate and tris buffers and allowed to stand at 4° for 24 hours, the pH of the mixtures were readjusted to 0.2 *M* pH 6.0 and the three sulfatase activities were determined.

SUMMARY

1. Sulfatases (arylsulfatase, glucosulfatase and cellulosepolysulfatase) in the liver extract of *Charonia lampas* have been separated from coexisting polysaccharases by selective precipitation of polysaccharases with trypaflavin and subsequently by selective adsorption of sulfatases on CM-cellulose,

DEAE-cellulose, or by electrophoresis.

Arylsulfatase, glucosulfatase and cellulosepolysulfatase were purified about 20-fold, 160-fold and 6-fold respectively, of the crude extract.

2. The properties of sulfatases were studied with the purified enzyme preparation. pH optimum of glucosulfatase and cellulosepolysulfatase were 5.8 and 6.2 respectively. Arylsulfatase, glucosulfatase and cellulosepolysulfatase were most stable at pH 5–6.5, pH 5.0 and pH 7.0 respectively at 4°.

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STUDIES ON CEPHALIN-CHOLESTEROL-FLOCCULATION REACTION

I. EFFECTS OF EGG YOLK LECITHIN ON THE REAGENT OF CEPHALIN-CHOLESTEROL-FLOCCULATION TEST

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In the original Hanger's method of cephalin-cholesterol flocculation test (*1*) (CCF test), crude cephalin from sheep brain was used as a component of the CCF reagent, but in Japan that from ox brain has also been utilized for the same purpose. Crude cephalins from various sourses were separated into five fractions by Folch in 1942 (*2*), the first fraction mainly consisting of inositol phospholipid, the third of phosphatidyl serine and the fifth of phosphatidyl ethanolamine, respectively. The second and fourth fractions gave no additional component other than those described above. Recently plasmalogens were also reported by Klenk (*3*, *4*) and others (*5*, *6*) to be a component of the fifth fraction above mentioned.

In spite of many informations about the chemical nature of cephalin, it is not yet certain whether a single component of cephalin fraction or some mixture of components would participate in CCF reaction. On the other hand, CCF reagents available on the market are known to differ widely in their flocculation activity according to their makers and more or less with batches of preparation. The inconstancy of reactivities among batches of preparation may lead to confusion of clinical evaluation of CCF test followed by loss of reliance on the test. These facts have hindered the CCF test from its broad application in Japan. The differences in reactivities have not yet been chemically explained. From the knowledge about cephalin mixture, however, it is assumed that any single fraction of cephalin may be the most potent in reactivity and others non-reactive or inhibitory. The differences in reactivities of reagents above mentioned may be attributed to the relative amount of the active component in cephalin preparation used in the test. If a single component is reactive, it may be possible properly to control its reactivity by means of admixture of a non-reactive or an antagonistical lipid matter in its purified state. As shown in the preliminary report (*7*), the third fraction designated by Folch as Fr-III (phosphatidyl serine fraction) was found to be the most reactive component, but CCF reagents prepared from only Fr-III seemed to be too

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sensitive for clinical purposes. It is well known that purified egg yolk lecithin is utilized as a controlling factor on the activity of cardiolipin-lecithin antigen in the case of serological diagnosis of syphilis (*8*). Phosphatidyl ethanolamine fraction has also a controlling capacity on cardiolipin antigen in the case of serological diagnosis of leprosy (*9*). This report concerns with a controlling effect of purified egg york lecithin on the reactivity of Fr-III in CCF test and a stabilizing effect on reagent suspension.

EXPERIMENTALS

Materials—Crude cephalins were prepared from pig brain and ox brain according to Klenk's method (*10*). Each crude cephalin was separated into five fractions using Folch's chloroform-alcohol fractionation method (*2*, *11*). The second and fourth fractions were discarded, and the first, third and fifth fraction were each tested as CCF reagent.

Egg yolk lecithin was prepared according to Pangborn's method (*12*).

The chemical analysis data of these samples were indicated in Table I.

TABLE I

Chemical Analysis of Phospholipids

	P (%)	N (%)	Serine (%)	Ethanolamine (%)
Pig-Brain Crude Cephalin	3.19	1.58	3.54	0.47
Pig-Brain Fraction I	4.20	2.90	3.54	0.55
Pig-Brain Fraction III	3.80	2.04	6.74	2.51
Pig-Brain Fraction V	3.00	1.74	2.45	5.21
Ox-Brain Crude Cephalin	3.13	1.92	—	—
Ox-Brain Fraction III	3.82	1.50	9.90	1.10
Egg Yolk Lecithin	3.86	1.67	—	—

Normal and pathological sera were fractionated by zone electrophoresis on starch. Zone electrophoresis were carried out under the following conditions: Veronal buffer pH 8.6, μ=0.05, 10 mA (about 2 mA/cm^2), 400 volts, temperature 4°. Each fraction of both sera eluted from blocks of starch was electrophoretically homogeneous, as shown in Fig. I.

CCF reactions were carried out according to Hanger's direction with CCF reagents which were dosed 12.5 mg. cephalin component to 37.5 mg. cholesterol for 60 persons. In the present testes the quantities of the lipid suspension and testing sera were the half of Hanger's original.

Analytical method—Total nitrogen was determined by micro-Kjeldahl method, phosphorus by King's method (*13*). Using the alkaline hydrolysates, serine and ethanolamine were examined by the method of Axelrod *et al.* (*14*). Paperchromatography for phospholipids was carrid out in asccending method on Toyo-Roshi No. 51 filter papers for 6 hrs, 18° using *n*-butanol-ethylenglycol-water (4 : 1 : 3) as solvent (*15*). Phosphorus and amino-nitrogen were detected by spraying Hanes-Isherwood reagent (*16*) for the former and ninhydrin-butanol solution for the latter. R_f values were shown

in Table II. The spot of 0.51–0.54 in R_f value represented phosphatidyl serine and 0.79–0.81 in R_f value phosphatidyl ethanolamine (*15*, *17*). Fr-III was therefore a mix-

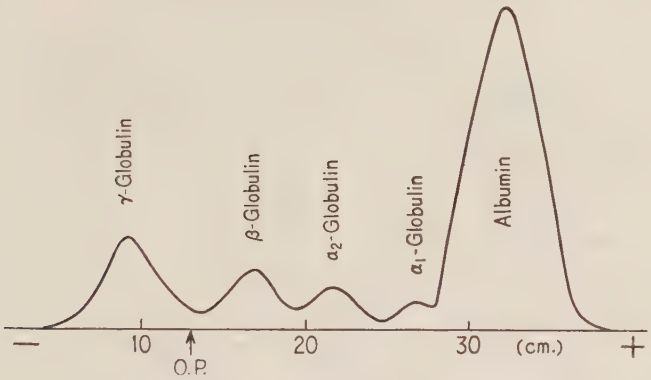

Fig. 1. Electrophoretic pattern of the mormal serum protein.

Table II

R_f Values of Cephalin Components

	Phosphorus		Amino Nitrogen	
Pig-Brain Crude Cephalin	0.54	0.80	0.53	0.79
Pig-Brain Fraction III	0.54	0.82	0.54	0.80
Pig-Brain Fraction V		0.82		

Solvent: Butanol-ethylenglycol-water (4 : 1 : 3) 18°, 6 hrs. in ascending method.

ture of phosphatidyl serine and phosphatidyl ethanolamine, but the former might be a major component judging from the chemical analysis shown in Table I. Pure phosphatidyl serine could not be prepared as described by Folch according to the solvent fractionation method (*11*).

RESULTS

Comparative observation of CCF reactions carried out, using Folch's fractions (Fr-I, Fr-III, Fr-V) of pig-brain cephalin, showed Fr-III to be the most reactive component (Table III). Ninety sera out of 93 tested were obtained from liver disease patients. These results were almost the same as those reported previously. However, it might be another problem what fraction would be appropriate for the clinical purposes, because the positive CCF reaction was coincided not always with cases of liver diseases.

Comparison of reagents prepared from Fr-III with the commercial products, Difco's reagent and Sumitomo's, in CCF reactions were indicated

in Table IV. From Table IV, it could be seen that Fr-III was too reactive in comparison with two commercial products and thymol flocculation test.

Table III

CCF Reaction of Cephalin Components

Reagent	Negative (%)[1)]	Positive (%)[1)]
Pig-Brain Crude Cephalin	15.2	84.8
Pig-Brain Fraction I	16.6	83.4
Pig-Brain Fraction III	0.6	99.4
Pig-Brain Fraction V	22.6	77.4

1) Expressed as mean value of 93 cases.

Table IV

Comparison of CCF Reaction by Fr-III Reagents with Those by Difco's and Sumitomo's Reagents

Reagent	Negative (%)	Positive (%)
CCF (Difco)[1)]	69.5	30.5
CCF (Sumitomo)[2)]	98.2	1.8
Ox-Brain Fr-II	30.0	70.0
Pig-Brain Fr-III	92.4	7.6
Thymol Flocculation	94.8	5.2

1) Lot No. 433789
2) Lot No. 1
300 sera obtained from the donors of blood bank. These donors are bleeded on an average twice by month.

From the experiences in cardiolipin-lecithin antigen for serodiagnosis of syphilis, in which the excess or lack of lecithin influenced much on the reactivity of antigen, the controlling effects of lecithin upon the reactivities of Fr-III would be expected. A series of CCF reactions using Fr-III or crude cephalin reagents containing various amounts of lecithin were carried out with sera from blood bank's donors. The results in the cases of pig brain Fr-III, ox brain Fr-III and crude cephalin were shown in Table V, VI and VII respectively.

According to Tables V, VI and VII, the controlling capacity of lecithin upon the reactivities of cephalins was recognizable and the amounts of lecithin to be added would vary for each preparation to obtain the same activity. From those results it was likely that the reactivity of crude cephalin was depressed more strongly by the same amounts of lecithin than

the cases of Fr-III. In those experiments the ratio of Fr-III to cholesterol

TABLE V

Controlling Effect of Lecithin on the Reactivity of Pig-Brain Fr-III Reagent

(250 cases)

Fr-III : Chol. : Lec.[1)]	Negative (%)	Positive (%)
1 : 3	10.4	89.6
1 : 3 : 0.05	16.7	83.3
1 : 3 : 0.1	39.8	60.2
1 : 3 : 0.2	83.3	16.7
1 : 3 : 0.3	87.7	12.3
1 : 3 : 0.4	93.8	6.2
1 : 3 : 0.5	99.0	1.0
1 : 3 : 1	100.0	0
Thymol Flocculation	85.5	14.5

1) Cholesterol and lecithin were abbreviated to Chol. and Lec. respectively.

44 Cases out of 250 were obtained from patients of liver diseases.

TABLE VI

Controlling Effect of Lecithin on the Reactivity of Ox-Beain Fr-III Reagent

(200 cases)

Fr-III : Chol. : Lec.	Negative (%)	Positive (%)
1 : 3	16.5	83.5
1 : 3 : 0.1	45.8	54.2
1 : 3 : 0.2	58.4	41.6
1 : 3 : 0.3	68.8	31.2
1 : 3 : 0.4	81.3	18.7
1 : 3 : 0.5	93.9	6.1
1 : 3 : 1	100.0	0
Thymol Flocculation	70.8	29.2

63 Cases out of 200 were obtained from the patients of liver disease.

and the amounts of two components were unchanged, though it could be

expected to prepare the more adequate reagent, if the ratio of those three

Table VII

Controlling Effect of Lecithin on the Reactivity of Ox-Brain Crude Cephalin Reagent

(200 cases)

Crude Cep. : Chol. : Lec.	Negative (%)	Positive (%)
1 : 3	16.5	83.5
1 : 3 : 0.05	58.5	41.5
1 : 3 : 0.1	69.0	31.0
1 : 3 : 0.15	83.5	16.5
1 : 3 : 0.25	89.8	10.2
1 : 3 : 0.5	100.0	0
1 : 3 : 1	100.0	0
Thymol Flocculation	91.8	9.2

22 Cases out of 200 were obtained from the patients of liver diseases.

components and their quantities were adjusted respectively (*18*).

In CCF reactions γ-globulin and albumin are important factors, of which the former acts to precipitate the lipid suspension and the latter to inhibit the precipitation according to Maclagen (*18*).

Table VIII

The Inhibitory Effects of Lecithin on the CCF Reaction with γ-Globulin

CCF reaction	Reagent / Serum No.	γ-Globulin (mg.) Pig-Brain Fr-III without lecithin	γ-Globulin (mg.) Pig-Brain Fr-III[1] with lecithin
−	Z-8	0.533	2.120
−	Z-10	0.400	1.600
−	Z-11	0.394	0.790
+	Z-17	0.105	0.470
##	Z-16	0.042	0.375
##	Z-18	0.038	0.300

1) Pig-Brain Fr-III : Chol. : Lec.—1 : 3 : 0.5.

Quantities in mg. of γ-globulin indicate the minimum to demonstrate ## positive reactions with a dose amount of CCF reagent.

For further investigation to secure the controlling effects of lecithin,

the interations between CCF reagent and serum protein fractions were compared in the presence and absence of lecithin. Normal and patients (liver diseases) sera were fractionated into albumin, α-, β- and γ-globulin by means of the zone electrophoresis described above. Each protein fraction dissolved in saline was mixed with the CCF reagent prepared with Fr-III and tests were made as in the case of whole serum. All samples of γ-globulins and β-globulins reacted with the CCF reagent, but it needed larger amounts of γ-globulin fractionated from normal sera than that from pathological sera to demonstrate positive reaction with the same amounts of CCF reagents as shown in Table VIII.

From the table, it was obvious that lecithin inhibited the precipitation of the CCF reagents, because in normal and pathological cases larger amounts of γ-globulin were needed to give positive reaction than the case with CCF reagents without lecithin.

The stabilizing effect of lecithin on reagent suspension was also examined. For this purpose, a comparative study of stored reagent suspensions was carried out with 50 sera, which was consisted of 30 cases of blood bank's doners, 13 cases of liver diseases and 7 cases of miscellaneous diseases. The stabilizing effect of lecithin on reagent suspension was recognized as shown in Table IX.

Table IX

Stabilizing Effect of Lecithin on the CCF Reagent Suspension (50 cases)[1]

	Fr-III Reagent[2]			Sumitomo's Reagent[3]		
	−	++	##	−	++	##
0	32	2	19	24	8	18
1 Day Storage	30	3	17	28	5	17
3 Days Storage	32	2	16	35	7	8
5 Days Storage	31	3	16	39	3	8
7 Days Storage	35	4	11	42	5	3

1) 30 cases out of 50 were obtained from doners of blood bank. 13 cases out of 50 were obtained from the patients of liver diseases and 7 cases out of 50 from the patients of miscellaneous diseases.

2) CCF Reagent ; Pig-Brain Fr-III : Chol. : Lec.—1 : 3 : 0.2.

3) Sumitomo Lot T-7.

The activity of pig brain Fr-III reagent was adjusted to that of Sumitomo's by mixing 1 part of Fr-III with 0.2 part of lecithin. When the suspensions of these reagents were stored in refrigerator, flocculates

deposited after 2 days in the case of Sumitomo's reagent, while after 6 days in the case of Fr-III reagent mixed with lecithin. Application of the supernatants resulted in the increase of cases of negative reaction.

DISCUSSION

For clinical purpose of CCF test, it is most important to successively apply the CCF reagent with constant potency. This could be prepared by dosing the most reactive component, Fr-III (phosphatidyl serine fraction) with antagonistical lipid, lecithin, in a proper ratio. Standardization of CCF reagent was carried out conveniently in comparison with Sumitomo's reagent such as indicated in Table X. Increase of the amounts of lecithin depressed the reactivity of CCF reagents as shown in Table V, VI, and VII. In these cases, the suitable amounts of lecithin might be 0.2–0.3 part to 1 part of Fr-III with the references of Thymol flocculation tests. But the CCF reagent used in the experiment indicated in Table VIII contained 0.5 part of lecithin to 1 part of Fr-III.

But standardization of CCF reagent should be performed, in comparison with the stable reference standard, which will be afforded by mixing the pure phospholipids such as prepared by synthesis or repeated column chromatography. Now, studies on the standard reagent and standard serum specimens are being carried out and will be reported later. The reactivity of phosphatidyl serine with serum proteins has not yet been explained. Combinations between cephalin and basic protein, *i.e.* salmine, globin (*20*) and clupein (*21*) in wide range of pH were reported. And interactions between fatty acid anions and serum albumins were also reported by

Table X

Inhibitory Effects of Various Serum Albumin on the Precipitation of γ-Globulin

CCF Reaction	Serum No.	γ-Globulin (mg.)			
		γ-Globulin without Albumin	γ-Globulin with Albumin from No. 1 Serum	γ-Globulin with Albumin from No. 2 Serum	γ-Globulin with Albumin from No. 3 Serum
–	No. 1	0.4[a)]	1.5[b)]	0.75[b)]	0.5[b)]
+	No. 2	0.25	3.0	0.75	0.75
##	No. 3	0.08	0.4	0.4	0.4

CCF Reagent; Pig-Brain Fr-III : Chol. : Lec. 1 : 3 : 0.5.

a) The minimum amounts of γ-globulin required to demonstrate ## positive reactions with a dose amount of CCF reagent.

b) The same as described above in the presence of 3 mg. of added albumins.

Micheel (*22*) and Goodman (*23*). According to these results and Chargaff's classification of lipoproteins forms (*24*) formed by combination of lipids and proteins, it is assumed that carboxyl group of phosphatidyl

serine may play an important role in CCF reaction.

Phosphatidyl ethanolamine might have a lower activity to precipitate serum proteins than phosphatidyl serine according to much less positive CCF reactions as shown in Table III. From these results and the role in cardiolipin-phosphatidyl ethanolamine antigen for serological reaction of leprosy (*9*), phosphatidyl ethanolamine might act in the same manner as lecithin. The controlling effect of lecithin on the reactivity of CCF reagent and the stabilizing effect on CCF reagent suspension may be essentially of the same kind. Serum albumin inhibited the precipitation of γ-globulin with CCF reagent prepared without lecithin addition as shown in Table X. Lecithin behaved similarly (Table VIII). From the analogy of properties of serum albumin on γ-globulin, and it appeared to act also as a protective colloid for phosphatidyl serine suspension.

SUMMARY

1. Phosphatidyl serine fraction prepared by Folch's method from crude cephalin was the most reactive factor in CCF reaction, as compared with other fractions.

2. Lecithin inhibited the precipitation of serum proteins or γ-globulin by phosphatidyl serine suspension. The CCF reagent with constant potency could be prepared by mixing of lecithin with phosphatidyl serine fraction in a proper ratio.

3. Lecithin also stabilized the CCF reagent suspension for a few days' storage.

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TERMINAL OXIDATION SYSTEM IN BAKER'S YEAST

III. SUBSTRATE-SPECIFICITY OF BAKER'S YEAST LACTIC DEHYDROGENASE

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During ealier investigation in Okunuki's laboratory (*1*, *2*), it was found that a preparation of baker's yeast lactic dehydrogenase dehydrogenates lactic acid, malic acid and reduced triphosphopyridine nucleotide (TPNH). The resulting electrons are transferred to externally added cytochrome c *via* cytochrome b_2 which is tightly bound to the enzyme. In the presence of the enzyme preparation, the reduction of cytochrome c by lactate and malate is optimal at pH 6.5 and 8.0, respectively. It was suggested in the previous paper (*1*) that because of this notable difference in the pH optima, the dehydrogenation of lactate and malate might be catalyzed by different dehydrogenases. These might have individual cytochrome b_2 moieties or a common one in some bound state of different kinds.

In 1928, Bernheim (*3*) found that an extract of baker's yeast catalyzed dehydrogenation of lactate and α-hydroxybutyrate. Later, Lehman (*4*), Dickens and Williamson (*5*) and Wieland (*6*) reported that a preparation of yeast lactic dehydrogenase dehydrogenates glycerate, 3-phosphoglycerate and α-hydroxybutyrate in addition to lactate. Therefore, the cytochrome b_2 which is tightly bound to yeast lactic dehydrogenase may have an important role in the dehydrogenation of many substrates in respiring yeast cells.

On the other hand, Boeri *et al.* (*7*) reported that their purest preparation of yeast lactic dehydrogenase did not reduce ferricytochrome c in the presence of any substrate other than L-lactate. They concluded that this enzyme was not an α-hydroxydase, a term which was once proposed by Barron (*8*).

In the present paper, it is shown that a fairly purified preparation of yeast lactic dehydrogenase can dehydrogenate α-hydroxybutyrate. The resulting electrons are transferred to externally added cytochrome c *via* cytochrome b_2 which is bound to the dehydrogenase. Some evidences will also be presented to show that both α-hydroxybutyrate and lactate are dehydrogenated by the dehydrogenase-moiety having the so-called group specificity and the resulting electrons are transferred to the same cytochrome b_2 moiety.

MATERIALS AND METHODS

Preparation of Crystalline Cytochrome c from Baker's Yeast—This was prepared as previously reported (*9*, *10*).

Preparation of Baker's Yeast Ferricytochrome c—Ferricytochrome c were prepared from crystalline yeast cytochrome c according to the method of Nozaki *et al.* (*10*).

Preparation of Baker's Yeast Lactic Dehydrogenase—This was prepared from baker's yeast according to the method of Bach *et al.* (*11*) and Yamanaka *et al.* (*1*) with slight modifications at the steps of disruption of the yeast cells, extraction of the enzyme from the disrupted cells and elution of the enzyme from calcium phosphate gel: Five kg. of compressed baker's yeast cells were kneaded with 500 ml. of ethylacetate. The resulting cellular fluid was mixed with 5 liters of 10 per cent aqueous NaCl. The mixture was stirred at room temperature (10°) for five hours; the pH was frequently adjusted to 7.0 with 5 *N* NaOH. And then the mixture was centrifuged in a Sharples centrifuge. The resulting supernatant was reserved for the preparation of cytochrome c. The cellular debris was resuspended in 5 liters of 1 per cent sodium lactate solution (adjusted to pH 7.5 with 5 *N* NaOH) and allowed to stand overnight in a refrigerator (5°). After centrifugation, the supernatant was dialyzed overnight against 50 liters of 0.5 per cent aqueous sodium lactate (pH 7.5) in a refrigerator. The solution was then treated with calcium phosphate gel. The gel was washed with 0.2 *M* sodium phosphate buffer, pH 7.0, until the supernatant fluid became colourless. The enzyme was eluted from the gel by the same buffer containing 20 per cent ammonium sulfate. Baker's yeast lactic dehydrogenase in its oxidized form was prepared by washing the enzyme preparation with cold 60 per cent saturated ammonium sulfate solution and then dialyzing it against 0.05 *M* sodium phosphate buffer, pH 7.0, for two hours in a refrigerator. Before use, a test was made to see if the oxidized preparation would reduce ferricytochrome c in the absence of substrate.

Assay of Activity of Baker's Yeast Lactic Dehydrogenase—The dehydrogenase was assayed by the method of Yamanaka *et al.* (*1*). Oxidation of lactate and α-hydroxybutyrate by the preparation was measured as oxygen uptake in the presence of phenazine methosulfate. This latter reagent has been used to measure succinic dehydrogenase activity by Singer *et al.* (*12*). In the phenazine methosulfate-assay of yeast lactic dehydrogenase, oxygen uptake was measured at 38° in a Warburg manometer. The reaction mixture contained the following components: 0.1 *M* sodium phosphate buffer, 20 μmoles of substrate at the same pH as the buffer and 0.1–0.4 mg. (dry weight) of enzyme protein; side chamber, 3.0 mg. of phenazine methosulfate. The total volume was adjusted to 2.0 ml. with distilled water. After 5 minutes equilibriation, the components were mixed and oxygen uptake was measured for first 5 minutes.

Heat Treatment of Enzyme—A suitable concentration of the oxidized enzyme was dissolved in 0.05 *M* of sodium phosphate buffer, pH 7.0, and incubated in the presence and absence of 0.001 *M* sodium lactate or α-hydroxybutyrate. The mixture was incubated at 35.5° for reduction of ferricytochrome c and at 43° for oxygen uptake by phenazine methosulfate. After incubation for suitable periods, aliquots were removed and immediately chilled in an ice-water bath. Their activity was assayed as soon as possible.

Chemical Reagents—DL-Sodium lactate and DL-sodium α-hydroxybutyrate were obtained from Wako Chemical Industries, Ltd.. L-β-Hydroxybutyrate was purchased from Nutritional Biochemicals Corporation, and β-hydroxypropionate from Tokyo Kasei Kogyo Co. Ltd.. Phenazine was kindly given by Dr. T. P. Singer. Phenazine methosulfate was

synthesized from phenazine by the method of Singer *et al.* (*12*).

RESULTS

Oxidation of Lactate and α-Hydroxybutyrate by Yeast Lactic Dehydrogenase in the Presence of Phenazine Methosulfate—Baker's yeast lactic dehydrogenase reduced certain dyes such as methylene blue (*1*, *3–7*, *13*) and compounds such as cytochrome c's from various sources (*1*, *2*, *6*, *7*, *11*). Moreover, with this enzyme there was oxygen consumption with lactate in the presence of phenazine methosulfate or methylene blue (*13*). A highly purified preparation of yeast lactic dehydrogenase dehydrogenates other substrates, such as malate, TPNH (*1*), glycerate or 3-phosphoglycerate (*3*, *4*). The electrons liberated from the substrates are transferred to dyes and cytochrome c. α-Hydroxybutyrate is so rapidly oxidized by the enzyme preparation that it is comparable with lactate as a substrate. The other substrates mentioned above are much slower oxidized. Therefore, lactate, and α-hydroxybutyrate were used as substrate to see if both were dehydrogenated due to group specificity towards α-hydroxymonocarboxylic acid of one dehydrogenase or by different dehydrogenases. Both lactate and α-hydroxybutyrate were oxidized by the preparation in the presence of phenazine methosulfate. With the above enzyme preparation, lactate was oxidized more rapidly than α-hydroxybutyrate. (Fig. 1). The oxygen uptake with either substrate was proportional to the enzyme concentration (Fig. 2).

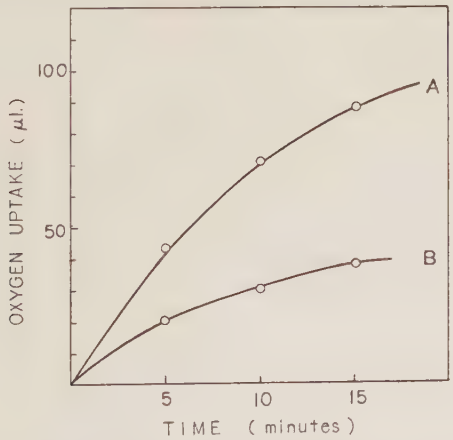

Fig. 1. Oxidation of lactate and α-hydroxybutyrate by baker's yeast lactic dehydrogenase with phenazine methosulfate.

The experimental conditions are described in the text. Curve A, lactate; Curve B, α-hydroxybutyrate.

As in the reduction of cytochrome c or methylene blue by lactate and α-hydroxybutyrate in the presence of phenazine methosulfate showed different

pH optima. The optimum was at pH 7.3 for lactate-oxidation and at pH 6.8 for α-hydroxybutyrate-oxidation (Fig. 3).

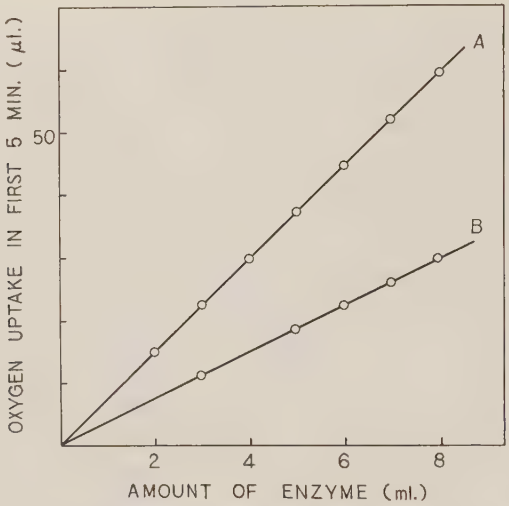

Fig. 2. Oxidation of lactate and α-hydroxybutyrate by yeast lactic dehydrogenase with phenazine methosulfate.

The experimental conditions are the same as for Fig. 1. Curve A, lactate; Curve B, α-hydroxybutyrate.

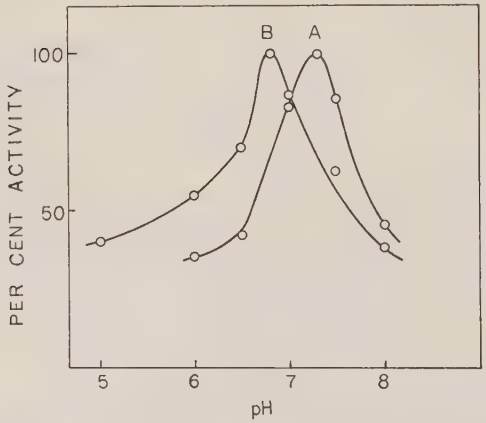

Fig. 3. Influence of pH on oxidation of lactate, and α-hydroxybutyrate by baker's yeast lactic dehydrogenase with phenazine methosulfate.

The experimental conditions are the same as for Fig. 1. Curve A, lactate; Curve B, α-hydroxybutyrate.

Reduction of Ferricytochrome c by Lactate, and α-Hydroxybutyrate in the Presence

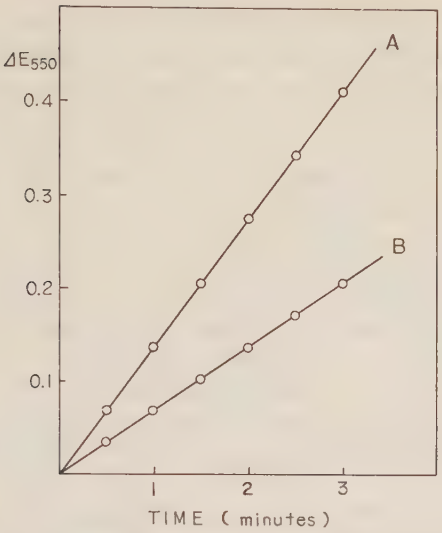

FIG. 4. Reduction of ferricytochrome c by lactate, and α-hydroxybutyrate in the presence of baker's yeast lactic dehydrogenase.

The components of reaction mixture were as follow; 500 μmoles of sodium phosphate buffer (pH 5.8), 30 μmoles of L-sodium lactate, and L-α-hydroxybutyrate, 270 μmoles of ferricytochrome c. Total volume, 3 ml. Temperature, 25°. Curve A, lactate; Curve B, α-hydroxybutyrate.

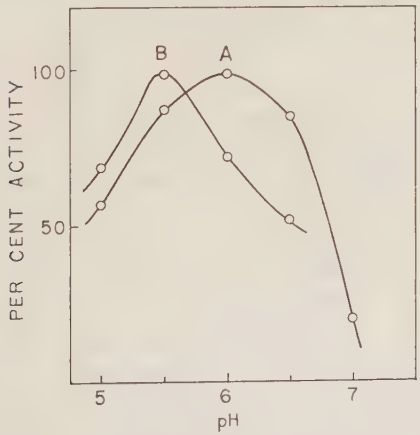

FIG. 5. Influence of pH on reduction of ferricytochrome c by lactate, and α-hydroxybutyrate in the presence of baker's yeast lactic dehydrogenase.

The experimental conditions are the same as for Fig. 4. Curve A, lactate; Curve B, α-hydroxybutyrate.

of Baker's Yeast Lactic Dehydrogenase—Cytochrome b_2 present in the yeast lactic dehydrogenase preparation was reduced on addition of α-hydroxybutyrate as rapidly as on addition of lactate. However, reduction of cytochrome b_2 attributed to dehydrogenation of α-hydroxybutyrate was less than that of lactate. If ferricytochrome c was present in the reaction mixture, electrons resulting from the dehydrogenation were transferred to it, perhaps *via* the cytochrome b_2 of the dehydrogenase. Reduction of cytochrome c by lactate and α-hydroxybutyrate differed in rate (Fig. 4). The pH optima for reduction of ferricytochrome c by lactate and α-hydroxybutyrate differed, *i. e.* pH 6.0 for lactate dehydrogenation and pH 5.5 for α-hydroxybutyrate (Fig. 5).

Activity of Baker's Yeast Lactic Dehydrogenase with β-Hydroxymonocarboxylic Acids—As previously shown, yeast lactic dehydrogenase catalyzed the oxidation of α-hydroxylic acids. However, β-hydroxylic acid could not act as substrate for this enzyme. There was no oxidation of β-hydroxypropionate or L-β-hydroxybutyrate in the presences of the enzyme and suitable electron acceptors.

Effect of Presence of Both Lactate, and α-Hydroxybutyrate on the Activity of Baker's Yeast Lactic Dehydrogenase Preparation—Using ferricytochrome c as an electron acceptor, lactate and α-hydroxybutyrate were simultaneously added

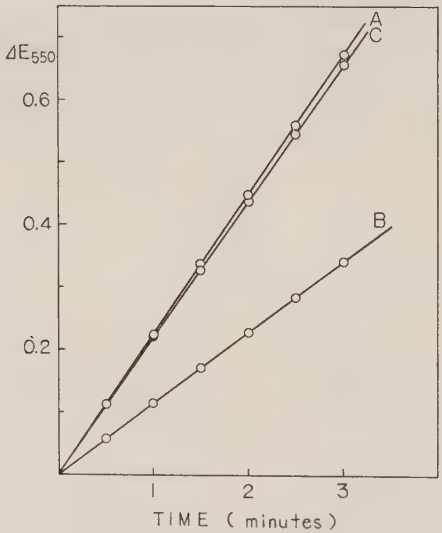

Fig. 6. Reduction of ferricytochrome c by lactate, α-hydroxybutyrate and lactate plus α-hydroxybutyrate.

The experimental conditions are the same as for Fig. 4, except that the pH of reaction mixture was 5.5. Curve A, lactate; Curve B, α-hydroxybutyrate; Curve C, lactate plus α-hydroxybutyrate.

to the yeast lactic dehydrogenase preparation. Each was added at a concentration of more than double the K_M (see blow). Even in the presence

of such a high concentration of each, lactate reduced ferricytochrome c more rapidly than α-hydroxybutyrate. The presence of both substrates did not increase their individual reduction rates (Fig. 6). Whether the yeast lactic dehydrogenase activity was assayed with phenazine methosulfate or with ferricytochrome c, no additive activity was observed in the presence of both substrates (Fig. 7).

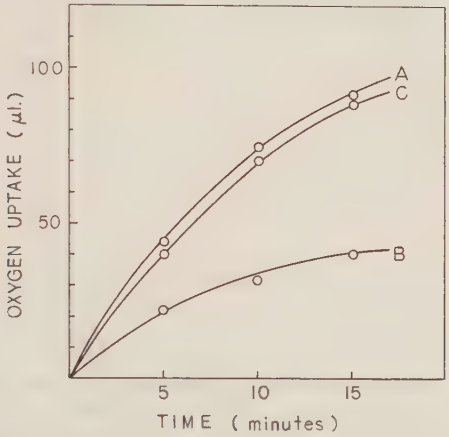

FIG. 7. Oxygen uptake with lactate, α-hydroxybutyrate, and lactate plus α-hydroxybutyrate in the presence of baker's yeast lactic dehydrogenase with phenazine methosulfate.

The experimental conditions are the same as for Fig. 1. Curve A, lactate; Curve B, α-hydroxybutyrate; Curve C, lactate plus α-hydroxybutyrate.

Kinetics of Reduction of Ferricytochrome c by Lactate, and α-Hydroxybutyrate in the Presence of Baker's Yeast Lactic Dehydrogenase—As mentioned above, in the presence of a high concentration of lactate and α-hydroxybutyrate, the baker's yeast lactic dehydrogenase preparation had the same reaction rate as with lactate alone. However, in the presence of a low concentration of substrate, there was additive activity.

Michaelis constant (K_M) for lactate and α-hydroxybutyrate were determined by the double reciprocal plot method. They were 6.4×10^{-4} *M* and 1.6×10^{-3} *M* for lactate and α-hydroxybutyrate, respectively, at pH 6.0 and 20° (Fig. 8 and 9).

As shown in Fig. 8, the slope in the presence of lactate was steeper than that in the presence of both lactate and α-hydroxybutyrate. However, the V_{max} was the same in both cases. A similar result was obtained for the α-hydroxybutyrate-cytochrome c reaction.

Effect of Various Reagents on Ferricytochrome c Reduction—Boeri *et al.* (7) reported that ferricytochrome c-reduction by yeast lactic dehydrogenase is inhibited by *p*-chloromercuribenzoate (PCMB). PCMB also inhibited the

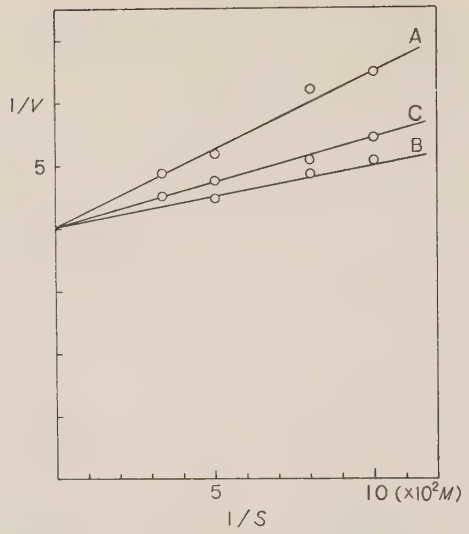

FIG. 8. K_M for lactate oxidation.

The experimental conditions are the same as for Fig. 4, except that the pH of reaction mixture was 6.0. Temperature, 20°. Abscissa, reciprocal lactate concentration; ordinate, reciprocal of the increment of optical density at 550 mμ per min. Curve A, lactate only; Curve B, +0.1 *M* α-hydroxybutyrate; Curve C, +0.01 *M* α-hydroxybutyrate.

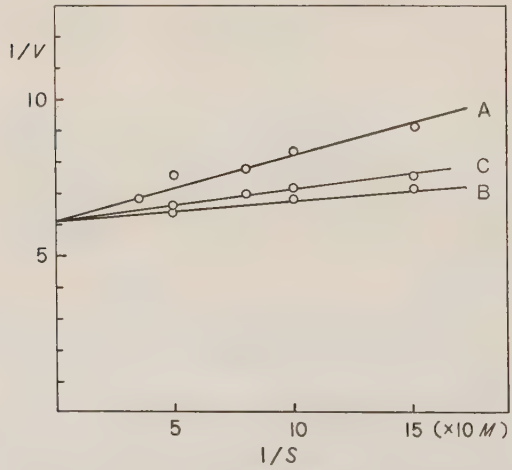

FIG. 9. K_M for α-hydroxybutyrate oxidation.

The experimental conditions are the same as for Fig. 8. Abscissa, reciprocal α-hydroxybutyrate concentration; ordinate, reciprocal of the increment of optical density at 550 mμ per min. Curve A, α-hydroxybutyrate only; Curve B, +0.005 *M* lactate; Curve C, +0.0025 *M* lactate.

α-hydroxybutyrate-ferricytochrome c reaction. PCMB at various concentration inhibited both activities to the same extent. After preincubation with 10^{-2} *M* cyanide for 20 min., both activities were inhibited while monoiodoacetate (MIA) had no effect (Table I).

TABLE I

Inhibition of the Lactate- and α-Hydroxybutyrate-Ferricytochrome c Reaction by Various Reagents

Reagent		Substrate	
		Lactate	α-Hydroxybutyrate
		Per cent inhibition[1]	
PCMB	3.3×10^{-5} *M*	100	100
,,	1.3×10^{-5} *M*	53	52
,,	1.0×10^{-5} *M*	35	33
,,	5.0×10^{-6} *M*	0	0
$-CN^-$	10^{-2} *M*	16[2]	22[2]
MIA	10^{-2} *M*	0	0

1) Enzyme activity was measured in *M*/15 sodium phosphate buffer, pH 7.5, with 0.1 *M* substrate. Temperature, 20°.

2) See text.

Effect of Heat Treatment on the Baker's Yeast Lactic Dehydrogenase Preparation—On mild heat treatment, baker's yeast lactic dehydrogenase in its oxidized form readily lost its ability to dehydrogenate lactate and α-hydroxybutyrate and to transfer the resulting electrons to cytochrome c (Table II). These two activities decreased in parallel. Loss of activity on heat treatment was prevented by the presence of substrate. This protective effect

TABLE II

Effect of Heat Treatment on a Preparation of Baker's Yeast Lactic Dehydrogenase

Temperature	Substrate	
	Lactate	α-Hydroxybutyrate
Degrees	Relative activity (per cent of control)	
Control	100 (0.155)[a]	100 (0.090)[a]
37[b]	71 (0.110)[a]	61 (0.055)[a]
42[b]	55 (0.085)[a]	50 (0.045)[a]
47[b]	39 (0.060)[a]	37 (0.033)[a]

a) Numerals in parentheses express increments in extinction at 550 mμ during initial 30 seconds of reaction.

b) The preparation in 0.02 *M* sodium phosphate buffer of pH 7.2 was heated at each temperature for five minutes and then immediately chilled in an ice-bath.

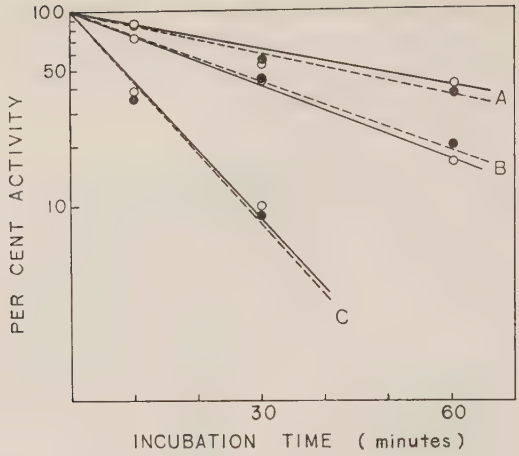

FIG. 10 Heat inactivation of ferricytochrome c reduction by baker's yeast lactic dehydrogenase.

The conditions for the experimental are given in the text. Activity was assayed as shown in Fig. 4. The dotted lines indicate relative activities for lactate and full lines those for α-hydroxybutyrate. Curve A, +lactate; Curve B, +α-hydroxybutyrate; Curve C, no substrate.

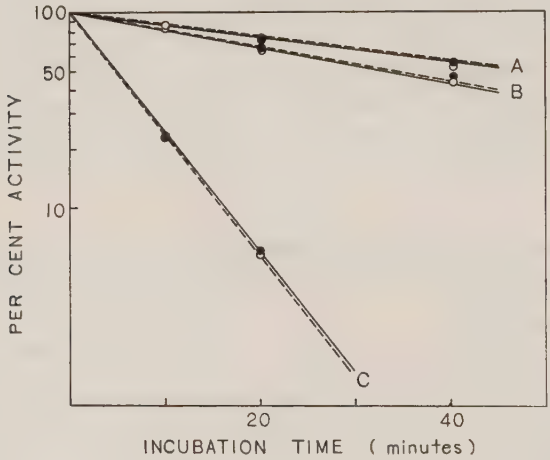

FIG. 11. Heat inactivation of oxidative activity of baker's yeast lactic dehydrogenase.

Experimental conditions are gived in the text. The activity was assayed as described in Fig. 1. The dotted lines indicate relative activities for lactate and full lines those for α-hydroxybutyrate. Curve A, +lactate; Curve B, α-hydroxybutyrate; Curve C, no substrate.

of the substrate has been applied to the preparation of this enzyme by many investigators. If the oxidation of lactate and α-hydroxybutrate were due to two different enzymes, it seems likely that lactate would only protect the lactate-oxidizing activity and α-hydroxybutyrate only that of α-hydroxybutyrate. If this were so, in the presence of lactate only lactate oxidation would remain, and *vice versa.* However, lactate protected both the lactate cytochrome c reaction and the α-hydroxybutyrate cytochrome c reaction to a similar extent (Fig. 10). The same was true with α-hydroxybutyrate to a lesser extent. This was demonstrated using the phenazine methosulfate assay method (Fig. 11).

Since both lactate and α-hydroxybutyrate have a hydroxyl group, it seemed possible that their protective action might be due to this group. Other hydroxylic acids, tartarate, citrate and glycolate had no protection and were not substrates for this enzyme.

DISCUSSION

The purest preparation of baker's yeast lactic dehydrogenase of Boeri and Tosi (*14*) contained one FMN residue, one heme group and eight non-heme iron atoms per 230,000 g. protein. These workers proposed that the hydrogen atoms liberated during dehydrogenation of lactate are transferred to the flavin of the enzyme, and thence *via* the eight non-heme iron atoms to the heme. The electrons are probably transferred from the heme of the enzyme to externally added cytochrome c. The purest baker's yeast lactic dehydrogenase preparation in this laboratory dehydrogenates α-hydroxybutyrate, malate and TPNH as well as lactate. The liberated electrons are transferred to the heme, since the α-peak of the absorption spectrum at 558 mμ of the reduced form increases (*1*). On the other hand, a cytochrome, cytochrome b_2, with the same absorption spectrum in the visible region as the lactic dehydrogenase can also be prepared from baker's yeast (*15*). Since this cytochrome has no flavin, no non-heme iron atoms, and no enzymatic activity, it seems likely that the cytochrome b_2 is only active when tightly bound to other dehydrogenase-moieties. Baker's yeast lactic dehydrogenase can reduce methylene blue and phenazine methosulfate as well as cytochrome c. It is therefore similar to the succinic dehydrogenase of Singer *et al.* (*16*). In a crude state succinic dehydrogenase reduced many chemical reagents, but only phenazine methosulfate in a pure state. This may mean that the enzyme becomes partially modified during purification, so that methylene blue can no longer act as an electron acceptor, *i. e.* baker's yeast lactic dehydrogenase in this partially modified form can reduce phenazine methosulfate but not methylene blue (*13*). Therefore in baker's yeast lactic dehydrogenase, there may be a lactic dehydrogenase-moiety and cytochrome b_2-moiety. When partially modified, the electron transporting system in the enzyme may be disrupted between these moieties. The preparation of baker's yeast lactic dehydrogenase dehydrogenated various substrates, as mentioned above. These substrates might be dehydrogenated by one or by

several dehydrogenase-moieties. At first the latter seemed likely since the dehydrogenation was optimal at different pH's with different substrates (*1*, *4*). For example, the lactate-methylene blue reaction was optimal at pH 5.5, and glycerate-methylene blue reaction, at pH 6.7. On the basis of these different optima it was suggested in the previous papers (*1*, *2*) that lactate and malate might be dehydrogenated by different enzymes. This suggestion was supported by the report of Boeri *et al.* (*7*) that a preparation of yeast lactic dehydrogenase could dehydrogenate only lactate.

Using lactate and α-hydroxybutyrate, the best of the substrates tested, this suggestion was re-evaluated by the following two methods: (1) Comparison of the ability of each substrate to prevent denaturation of the enzyme. (2) Comparison of oxidative activity of the enzyme with each substrate individually and with a mixture of the two substrates. Contrary to the conclusion of the previous papers, experiments showed that lactate and α-hydroxybutyrate are oxidized by a same dehydrogenase. Meister (*17*) also reported that lactic dehydrogenase from rabbit heart muscle can dehydrogenate various substrates besides lactate and that the pH optima for each substrate differed. Since substrates other than lactate and α-hydroxybutyrate are very slowly oxidized by baker's yeast lactic dehydrogenase, it might be difficult to repeat these experiments with other substrates. However, the cytochrome b_2 bound to the enzyme is almost completely reduced in the presence of a high concentration of lactate and all of the substrates, which are, except TPNH, α-hydroxylic acids. For the reason, it is concluded that baker's yeast lactic dehydrogenase is probably group specific, and electrons may be transferred from the dehydrogenase moiety to the cytochrome b_2 moiety *via* the flavin bound to the enzyme. Therefore, baker's yeast lactic dehydrogenase might be called α-hydroxycarboxylic dehydrogenase. The problem of the dehydrogenation of TPNH still remains unsolved.

The difference in optimal pH for the oxidation of the various substrates by yeast lactic dehydrogenase might be due to the differences in the pattern of active sites on the enzyme protein or to those in dissociation of these substrates at different pH's.

SUMMARY

1. Baker's yeast lactic dehydrogenase can utilize ferricytochrome c and phenazine methosulfate as electron acceptors in the oxidation of lactate and α-hydroxybutyrate.

2. Lactate is oxidized twice as rapidly as α-hydroxybutyrate. In the reduction of ferricytochrome c, optimum pH's are at 6.0 for lactate and 5.5 for α-hydroxybutyrate. With phenazine methosulfate, optima are at pH 7.3 and 6.8, respectively.

3. There is no summation of activity in the presence of both lactate and α-hydroxybutyrate during reduction of ferricytochrome c or oxygen uptake with phenazine methosulfate.

4. K_M values for lactate and α-hydroxybutyrate are $6.4\times10^{-4}M$ and $1.6\times10^{-3}M$, respectively, at pH 6.0, and 20°.

5. Heat treatment decreases the activity towards both substrates in parallel.

Results suggest that baker's yeast lactic dehydrogenase catalyzed the oxidation of both lactate and α-hydroxybutyrate.

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ADDENDUM

In 1954, Appleby and Morton (*1*) succeeded in crystallizing baker's yeast lactic dehydrogenase. However, we could not obtain details of their procedure and the enzymatic properties until last year. Recently, they (*2*, *3*) reported that crystalline yeast lactic dehydrogenase catalyzed the oxidation of α-hydroxy-*n*-butyrate as well as that of lactate. Inhibition of α-hydroxy-*n*-butyrate on lactate oxidation appeared at highest concentration of substrate in our experiments. They reported that L-malate did not act as substrate for baker's yeast lactic dehydrogenase, but inhibited the oxidation of L-lactate.

It will be discussed that the dehydrogenation of malate and the inhibition on the oxidation of L-lactate in a subsequent paper.

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STUDIES ON THE REACTIONS OF METMYOGLOBIN WITH SODIUM AZIDE AND POTASSIUM THIOCYANATE

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Generally, it has been assumed that the Bohr effect which is observed in the oxygenation reaction of hemoglobin may be due to the ionization of the heme linked acid groups. These groups are thought to be localized close to the heme moiety of the hemoglobin molecule. Wyman (*1*) suggested that these acidic groups might be the imidazole NH of the histidine residues of the protein to which hemes are attached. Coryell and Pauling (*2*) have interpreted the mechanism of the Bohr effect in terms of the influence of resonance of imidazole ring on acid strength of imidazole NH. Myoglobin has been assumed to have the same amino residue for the combination with heme as hemoglobin (*3*). However, myoglobin, in sharp contrast to hemoglobin, shows no Bohr effect (*4*).

The present investigation was therefore undertaken to further elucidate the identity of the heme linked acid group of myoglobin using the binding properties of metmyoglobin with sodium azide and potassium thiocyanate.

EXPERIMENTALS

1. Crystalline metmyoglobin was prepared according to the method of Tsushima and Okazaki (*5*) from equine heart muscle and was dissolved in appropriate buffer solutions. The concentration of a metmyoglobin solution was determined spectrophotometrically by the cyanide-metmyoglobin method using milli-molar extinction coefficient of 11.3 at 540 mμ (*6*).

2. Sodium azide and potassium thiocyanate were commercial preparations and they were used after dissolving in appropriate buffer solutions.

3. Buffers used were phosphate, borate and carbonate, and in all cases the ionic strength was adjusted to 0.17 with sodium chloride.

4. The spectrophotometric measurements were made with a photoelectric spectrophotometer Model EPB-V of Hitachi Co. and pH was measured with a glass electrode pH-meter of Toyo-Rika Co.

RESULTS

I. Reactions of Metmyoglobin with Sodium Azide and Potassium Thiocyanate —As shown in Fig. 1, the spectrum of metmyoglobin is converted to those of azide-metmyoglobin and thiocyanate-metmyoglobin in the presence of excess sodium azide and potassium thiocyanate, respectively. Since the

isosbestic points are clearly demonstrated at 519 and 613 mμ in Fig. 1 A and 509 and 610 mμ in Fig. 1 B, it is apparent that each of these reaction mixtures consists of two components, free metmyoglobin and azide-metmyoglobin or

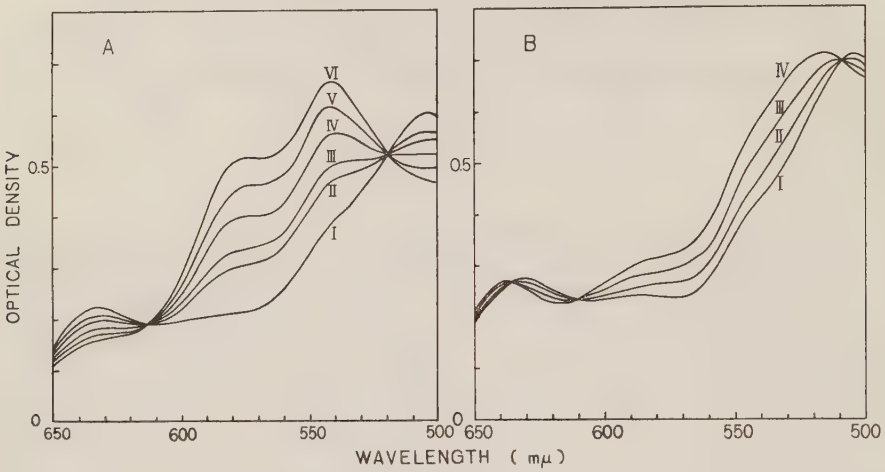

Fig. 1. Absorption spectra of metmyoglobin in the presence of various concentrations of sodium azide (A) and potassium thiocyanate (B). All measurements were made in phosphate buffer, pH 7.0, ionic strength, 0.17 and at 20°. Concentrations of metmyoglobin were 6.15×10^{-5} *M* (A) and 7.38×10^{-5} *M* (B), respectively.

(A) Concentrations of sodium azide (*M*): I; 0, II; 2.3×10^{-5}, III; 4.6×10^{-5}, IV; 8.9×10^{-5}, V; 1.7×10^{-4}, VI; 1.0×10^{-3}, 2.0×10^{-3}.

(B) Concentrations of potassium thiocyanate (*M*); I; 0, II; 1.6×10^{-3}, III; 6.0×10^{-3}, IV; 1.0×10^{-1}, 2.0×10^{-1}.

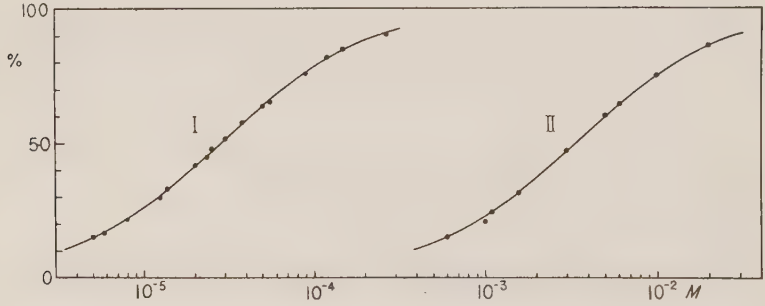

Fig. 2. Dependence of metmyoglobin complex formation upon the concentrations of sodium azide and potassium thiocyanate. Fraction of metmyoglobin complex was calculated from the results shown in Fig. 1.

Curve I: azide-metmyoglobin formation.

Curve II: thiocyanate-metmyoglobin formation.

thiocyanate-metmyoglobin, respectively. Based on the absorbancy changes at a definite wavelength, an attempt was made to calculate the dependence

of the spectral changes upon the sodium azide and potassium thiocyanate concentrations. By plotting the percentage formation of azide-metmyoglobin or thiocyanate-metmyoglobin against the logarithm of the concentrations of free sodium azide or potassium thiocyanate in the reaction mixtures, sigmoid curves of first-order reaction were obtained as shown in Fig. 2.

The apparent dissociation constants (K′), which are the concentrations of azide and thiocyanate giving half maximal saturation of metmyoglobin complexes, were calculated to be 2.8×10^{-5} and 3.4×10^{-3}, at pH 7.0 and 20°, respectively.

II. Effects of pH on the Reaction of Metmyoglobin with Sodium Azide and Potassium Thiocyanate—The effects of pH on these reactions were studied by changing the pH values from 6.0 to 10.0 at constant temperature. Each of the dissociation curves obtained at various pH values coincides with that of first order reaction. The affinity of metmyoglobin for azide or thiocyanate, however, decreases as the pH values increase. Plots of the pK' values against pH are shown in Fig. 3.

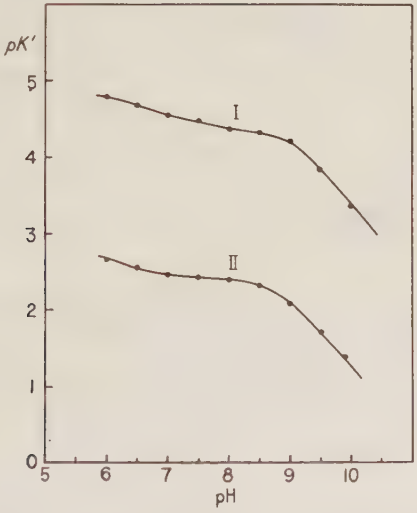

Fig. 3. Effects of pH on pK' values (ionic strength, 0.17, at 20°).
Curve I: the reaction of metmyoglobin with sodium azide.
Curve II: the reaction of metmyoglobin with potassium thiocyanate.

III. Effect of Temperature on the Reaction of Metmyoglobin with Sodium Azide and Potassium Thiocyanate—The effect of temperature on metmyoglobin complex formation was examined over a wide pH range. In Fig. 4, the pK' values calculated at different temperatures by means of the method similar to that mentioned above, were plotted against the reciprocals of the absolute

temperatures. In both reactions a linear relationship was obtained at every pH.

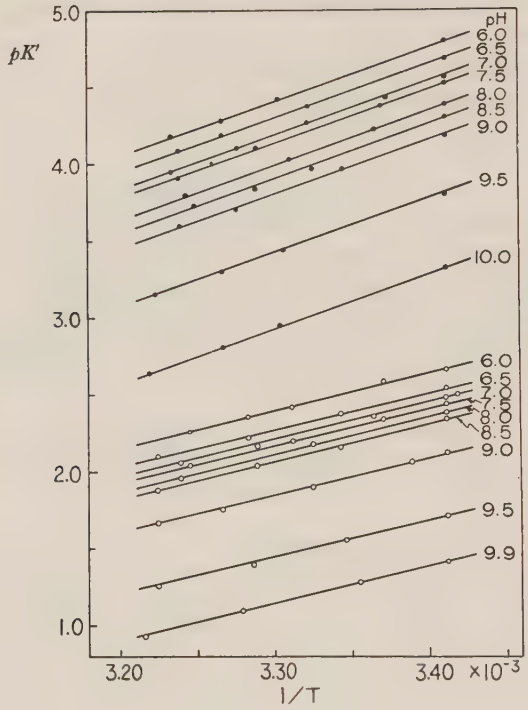

FIG. 4. Effects of temperature on *pK'* values.
—●— : the reaction of metmyoglobin with sodium azide.
—○— : the reaction of metmyoglobin with potassium thiocyanate.
The measurements were made at various pH values indicated.

From these results, the values of ΔH, ΔF and ΔS for the reactions of metmyoglobin with sodium azide and potassium thiocyanate were calculated as shown in Table I. For comparison the thermodynamic constants for the other reactions of hemoglobin and myoglobin reported by many investigators (*4*, *7–10*) are included in Table I, although they were not obtained under the same conditions.

IV. Properties of Heme Linked Acid Group of Myoglobin—The variations of *pK'* values in the pH range from 6.0 to 8.0, shown in Fig. 3,could be explained by the participation of heme linked acid group in these reactions. The change in slope in the pH range from 8.0 to 10.0 could be explained by the effect of ionization of the heme iron atom of metmyoglobin molecule upon these reactions. In addition to these factors, the participation of

ionization of the ligands in these reactions should be also considered.

TABLE I

Comparisons of Thermodynamic Data for Myoglobin and Hemoglobin Reactions

	ΔF (Kcal/mol)	ΔH (Kcal/mol)	ΔS (cal/mol/deg.)	references
N_3-metMb	− 6.10	−16.45	−35.3	the present
SCN-metMb	− 3.31	−10.56	−24.7	paper
N_3-metHb	− 7.19	−14.28	−22.8	(7)
SCN-metHb	− 2.64	− 9.70	−22.8	
Mb-O_2	− 7.5	−16.4	−30.0	(4, 8, 9)[a]
Mb-CO	− 9.2	−20.0	−36.2	
Hb-O_2 (first)	—	−12.1	−22.2	(9, 10)[b]
Hb-O_2 (last)	—	− 5.1	+13.0	

a) Calculated by George (*9*) from data given by Theorell (*4*) and Millikan (*8*)

b) Calculated by George (*9*) from data given by Roughton *et al.* (*10*).

Now assuming that myoglobin has only one heme linked acid group, as assumed by George and Hanania (*11*), the following equations, 1 to 5, may be proposed for the reaction mechanism of metmyoglobin with azide or thiocyanate.

$$\mathrm{Mb\cdot Fe^{III}\cdot H_2O + X^- \underset{k'}{\overset{k}{\rightleftharpoons}} Mb\cdot Fe^{III}\cdot X^- + H_2O} \qquad 1.$$

$$\mathrm{Mb\cdot Fe^{III}\cdot H_2O \overset{K_{Fe}}{\rightleftharpoons} Mb\cdot Fe^{III}\cdot OH^- + H^+} \qquad 2.$$

$$\mathrm{{}^+HMb\cdot Fe^{III}\cdot H_2O \overset{K_r}{\rightleftharpoons} Mb\cdot Fe^{III}\cdot H_2O + H^+} \qquad 3.$$

$$\mathrm{{}^+HMb\cdot Fe^{III}\cdot X^- \overset{K_p}{\rightleftharpoons} Mb\cdot Fe^{III}\cdot X^- + H^+} \qquad 4.$$

$$\mathrm{HX \overset{K_{HX}}{\rightleftharpoons} X^- + H^+} \qquad 5.$$

In these equations $\mathrm{Mb\cdot Fe^{III}\cdot H_2O}$ and $\mathrm{{}^+HMb\cdot Fe^{III}\cdot H_2O}$ represent acidic metmyoglobin and acidic metmyoglobin with the heme linked acid group in its conjugated basic form, respectively. HX is the protonated form of azide or thiocyanate. The velocity constants for the reaction of equation 1 are expressed as k and k', and the dissociation constants for the reactions of equations 2 to 5 are expressed as K_{Fe}, K_r, K_p and K_{HX}, respectively. Then the overall reaction is derived as follows:

$$K' = \frac{(K_r+\mathrm{H})\cdot K_p\cdot(K_{Fe}+\mathrm{H})\cdot(K_{HX}+\mathrm{H})}{K_r\cdot(K_p+\mathrm{H})\cdot \mathrm{H}\cdot K_{HX}}\cdot\frac{k'}{k} \qquad 6.$$

$$\frac{(K_r+\mathrm{H})\cdot K_p\cdot(K_{Fe}+\mathrm{H})}{K'\cdot K_r\cdot(K_p+\mathrm{H})\cdot \mathrm{H}}\cdot\frac{K_{HX}+\mathrm{H}}{K_{HX}} = \text{constant.} \qquad 7.$$

where K' represents the apparent dissociation constant, which was experimentally obtained above for the reaction of metmyoglobin with azide or thiocyanate, and H represents the hydrogen ion concentration.

For the analyses of the overall reactions, the dissociation constants of the heme iron atom and of the hydroazoic acid had to be determined precisely by means of the spectrophotometry and acid-base titration. The results were shown in Fig. 5. From these data the values of K_{Fe} and K_{HN_3} at 20° were calculated to be $10^{-9.02}$ and $10^{-4.65}$, and ΔH for the ionizations to be -5.2 Kcal/mole and -4.9 Kcal/mole, respectively.

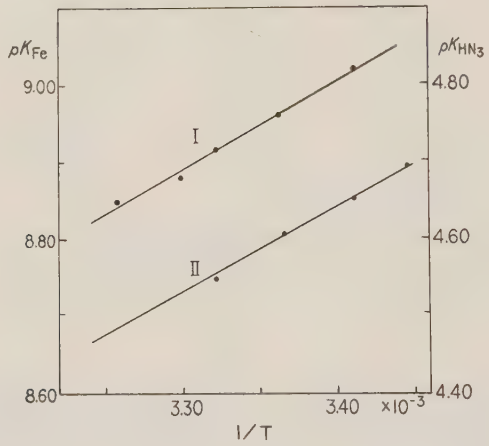

FIG. 5. Effects of temperature on pK_{Fe} and pK_{HN_3}.
I: pK_{Fe}, II: pK_{HN_3}.

Based on all experimental results, the dissociation constants of the heme linked acid group of metmyoglobin and its derivatives, expressed as K_r and K_p, respectively, can be calculated by using equation 7. In this way, the K_r and K_p values at 20° were calculated to be $10^{-6.11}$ and $10^{-6.63}$, respectively, in the case of the reaction of metmyoglobin with azide.

In the case of the reaction with thiocyanate, calculations become easier because thiocyanate is a strong acid, so that the analysis of this reaction can be done with equation 8 substituting for equation 7.

$$\frac{(K_r+H)\cdot K_p\cdot(K_{Fe}+H)}{K'\cdot K_r\cdot(K_p+H)\cdot H}=\text{constant.} \qquad 8.$$

Then $K_r=10^{-6.11}$ and $K_p=10^{-6.56}$ at 20° were given for the reaction with thiocyanate.

The temperature dependencies of K_r and K_p in both reactions can be calculated in a similar manner by using the constants obtained at various temperatures. The relationships between the pK_r and pK_p values and the reciprocals of the absolute temperatures were given in Fig. 6. The thermodynamic constants for the ionization of the heme linked acid groups of metmyoglobin and its derivatives thus obtained are shown in Table II. As

may be seen in this table, the pK_r value of the heme linked acid group of metmyoglobin is increased by about 0.5 when the ligand, azide or thiocyanate, combines with the heme iron atom of metmyoglobin.

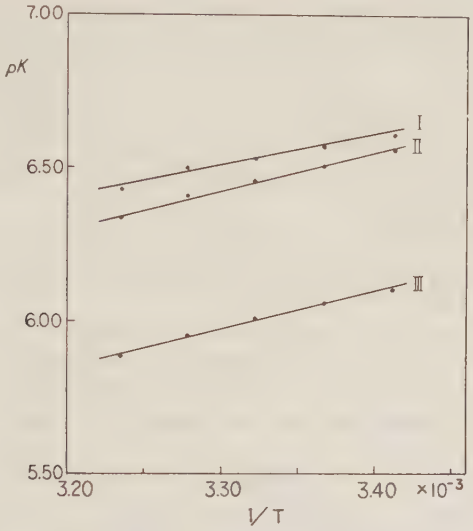

Fig. 6. Effects of temperature on the ionization constants of heme linked acid groups of metmyoglobin and its complexes. (see K_r and K_p in the text)
I: azide-metmyoglobin.
II: thiocyanate-metmyoglobin.
III: metmyoglobin.

Table II

Thermodynamic Data for the Ionization of the Heme Linked Acid Group of Metmyoglobin and its complexes

	T(°K)	pK	ΔF (Kcal/mol)	ΔH (Kcal/mol)	ΔS (cal/mol/deg.)
metMb	293	6.11[a)]	− 8.19	− 5.96	+ 7.61
N_3-metMb	293	6.63[b)]	− 8.89	− 5.20	+12.59
SCN-metMb	293	6.56[b)]	− 8.80	− 5.70	+10.58

a) expressed as pK_r and *b*) pK_p in the text.

Since the pK_r and pK_p values of the heme linked acid group obtained are close to 6.0 and all of the ΔH values for the ionizations are about 6 Kcal/mole, it can be assumed that the heme linked acid group in myoglobin may also be the imidazole group of histidine as in hemoglobin.

DISCUSSION

1. Although the heme linked acid group of myoglobin is assumed to be the imidazole group of histidine, there are still many aspects of this problem that are not fully understood. George and Hanania (*11*) have supported the histidine theory, on the basis of their calculations of the *pK* values of such groups in various derivatives of myoglobin. On the other hand, Theorell and Ehrenberg (*12*) have proposed that the group to which the myoglobin iron atom is attached is not histidine but a more negatively charged group.

Based on the thermodynamic data obtained from the present experiments, it may be proposed that the heme linked acid group of myoglobin is the imidazole residue of a histidine.

As far as is known, the variations of pK' values in the pH range 6.0 to 8.0 in the reactions of metmyoglobin with azide and thiocyanate (see Fig. 3) closely resemble those in the methemoglobin reactions reported by Jung *et al.* (*13*). This fact suggests that the heme linked acid groups of metmyoglobin and methemoglobin should be affected to the same extent in their ionizations when the heme iron atoms are bound by ligands. At least in these cases, there are no such differences between the reaction of metmyoglobin and that of methemoglobin with respect to the heme linked functions, as demonstrated in the oxygenations of both proteins in their reduced forms.

Considering these facts, the mechanism of the Bohr effect can not be elucidated through the changes of electronic structures of the acid groups alone. As proposed by Wyman (*14*), modifications in secondary structure of hemoglobin molecule during the functioning of hemoglobin, may largely contribute in some way to the mechanism of the Bohr effect.

2. Coryell and Pauling (*2*) proposed the theory that the ionizations of heme linked acid groups in hemoglobin and myoglobin derivatives may depend on the bond types of their heme iron atoms. However, this has been criticized by George (*11*) on the basis of his comparative experiments on various myoglobin derivatives.

In azide-metmyoglobin the bond type of heme iron atom is assumed to be covalent differing from ionic bond in thiocyanate-metmyoglobin (*9*). As shown in Table II, however, the ionization constant of the heme linked acid group in azide-metmyoglobin is not significantly different from that in thiocyanate-metmyoglobin. This fact gives support the George's argument and indicates that the ionizations of the heme linked acid groups should not depend on the bond types of the heme iron atoms.

SUMMARY

1. The thermodynamic constants for the reactions of metmyoglobin with sodium azide and potassium thiocyanate were determined as follows:

a) for the reaction with azide. (pH 7.0, at 20°) $\Delta H = -16.45$ Kcal/mole, $\Delta F = -6.10$ Kcal/mole, $\Delta S = -35.3$ cal/mole/deg.

b) for the reaction with thiocyanate. (pH 7.0, at 20°) $\Delta H = -10.56$ Kcal/mole, $\Delta F = -3.31$ Kcal/mole $\Delta S = -24.7$ cal/mole/deg.

ΔS values for both reactions did not differ significantly from the values which had been determined by other workers for various reactions of hemoglobin and myoglobin.

2. The dissociation constants for the heme linked acid groups of metmyoglobin, azide-metmyoglobin and thiocyanate-metmyoglobin were calculated to be $10^{-6.11}$, $10^{-6.63}$ and $10^{-6.56}$ at 20°, respectively. The heats of dissociation of these groups were calculated to be about 6 Kcal/mole in every case, indicating that the heme linked acid group of myoglobin might be the imidazole group of a histidine.

3. Some speculations were made on the mechanism of the Bohr effect.

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SULFUR METABOLISM IN HIGHER PLANTS

II. THE EFFECT OF SULFITE ON THE METABOLISM OF SULFATE AND ITS CONVERSION INTO ORGANIC FORM IN EXCISED LEAVES

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It has long been considered that sulfite is an intermediate in the biosynthetic pathway of cysteine from sulfate, and nutritional studies using bacterial mutants strengthened the hypothesis (*1*, *2*, *3*). Cowie and his associates carried out isotopic work on the sulfate metabolism of *Escherichia coli* and obtained results which might coincide with the above hypothesis (*4*). Shepherd reported results supporting this hypothesis using acetone powder extract of microorganisms (*5*). However, there has been very little work along this hypothesis using higher plants. Thomas and his group studied the effect of sulfite on the metabolism of sulfur in alfalfa and sugar beets, but their observation did not coincide with the above hypothesis and they could only conclude that sulfite absorbed by the plants might be oxidized to sulfate and next converted to some organic sulfur compounds (*6*). Accordingly a more thorough study on the role of sulfite is needed. The present paper describes the effects of sulfite on the metabolism of sulfate and its conversion into organic form in excised mung been leaves.

MATERIALS AND METHODS

Materials—S^{35}-labeled sulfite was prepared from S^{35}-labeled $BaSO_4$ following Johnson and Huston's method (*7*), except that the produced sulfite was trapped in 1 per cent ethylenediaminetetraacetate in order to prevent autoxidation. The resulting solution was neutralized to the phenolphthalein-endpoint with 1*N* HCl just before use. L-Cysteine-sulfinic acid, L-cysteic acid and L-cysteine-S-sulfonic acid were prepared from L-cystine by Lavine's, Shinohara's and Clarke's methods, respectively (*8*, *9*, *10*).

Mung beans were cultivated for 7 to 10 days and the primary leaves were used for all experiments.

Determination of the Incorporation of Sulfate-sulfur into Organic Sulfur—The leaves were incubated in a solution of S^{35}-labeled sulfate and the radioactivity of sulfate-S^{35} and total S^{35} in the incubated leaves was determined as described previously (*11*). The intensity of incorporation of sulfate-sulfur into organic sulfur was calculated from those values given in the previous paper (*11*).

Determination of the Incorporation of Sulfite-sulfur into Organic Sulfur—One g. of the excised

leaves was incubated for 3 hours at 30° under reduced pressure in 20 ml. of a solution containing labeled sulfite at a concentration of 1–5 μc per ml. and $5\times10^{-3}M$ non-labeled sulfite. After washing the incubated leaves with 0.1N iodide solution, they were homogenized with the same solution as used for washing. The resulting homogenate was divided into acid-soluble and acid-insoluble fractions by treating with 10 per cent trichloroacetic acid solution. Washing was repeated three times with 5 per cent trichloroacetic acid solution. The radioactivity of sulfate-S^{35} and total S^{35} was measured by the method described in the previous paper (*11*) except that organic sulfur was oxidized to sulfate sulfur by Pirie's method (*12*.)

Determination of the Incorporation of Sulfate-sulfite into sulfite-sulfur—After incubating 3 g. of the leaves in the medium containing S^{35}-labeled sulfate and $3\times10^{-2}M$ non-labeled sulfite, they were homogenized with 4 ml. of 1N NaOH and 30 mg. of Na_2SO_3. Then, the homogenate was warmed on a water bath by adding 4N HCl. Evolved sulfite was trapped in an alkaline solution and was oxidized to sulfate by the addition of iodine solution. After precipitating sulfate with $BaCl_2$ as $BaSO_4$, it was measured for radioactivity.

Extraction of Labeled Sulfur-containing Organic Compounds—Five g. of the incubated leaves were homogenized with 2 ml. of 1N KOH (or 1N NaOH when acid insoluble fractions were counted). The resulting homogenate was mixed with 10 ml. of 10 per cent $HClO_4$. The acid-soluble fraction, which was separated from the acid-insoluble fraction by centrifugation, was neutralized with 10 per cent KOH and kept overnight in an ice box. After filtration of the resulting $KClO_4$ precipitate, the supernatant was concentrated *in vacuo* at 25–30°. Simultaneously, the acid-insoluble fraction was washed repeatedly with 5 per cent trichloroacetic acid solution in order to remove $HClO_4$, and hydrolyzed finally in 6N HCl at 100°. The hydrolysate was concentrated under reduced pressure until HCl was removed completely.

RESULTS

Effect of Sulfite on the Incorporation of Sulfate-sulfur into Organic Compounds—Provided that sulfite is an intermediate in the biosynthetic pathway of sulfate into sulfur-containing organic compounds, it will be presumed that the

TABLE I

Effect of Non-labeled Sulfite on Incorporation of Sulfate-S^{35} into Organic Conmpounds in Excised Leaves

Infiltrated substances	Intensity of incorporation (%)[3]
S^{35}-labeled sulfate[1]	32
S^{35}-labeled sulfate+non-labeled sulfate[2]	7
S^{35}-labeled sulfate+non-labeled sulfite[2]	0

1) The solution did not contain non-labeled sulfate. The leaves were incubated in the solution at the concentration of 3 μc per ml.

2) In each case, the excised leaves were incubated in a solution containing S^{35}-labeled sulfate and $3\times10^{-2}M$ non-labeled sulfate or sulfite.

3) The values show the amount of S^{35} in organic compounds in percentage of the total S^{35} absorbed.

conversion of sulfate-S^{35} into organic form may be inhibited if non-labeled sulfite is infiltrated into the excised leaves together with S^{35}-labeled sulfate. Some experiments were carried out on the basis of this proposition. As shown in Table I, S^{35} was not incorporated into organic compounds, when the excised leaves were incubated in the medium containing S^{35}-labeled sulfate and $3\times10^{-2}M$ non-labeled sulfite. Since non-labeled sulfate at the same concentration as that of sulfite did not stop the conversion of sulfate-S^{35} into organic form on a large scale, inhibitory effect of sulfite can not be explained by the dilution of sulfate-S^{35} which was caused by sulfate formed from sulfite in the excised leaves.

Conversion of Sulfate-sulfur into Sulfite-sulfur—If sulfate-sulfur is metabolized to sulfur-containing organic compounds through sulfite-sulfur, it will be postulated that, when S^{35}-labeled sulfate and non-labeled sulfite are infiltrated together into the excised leaves, a part of sulfate-S^{35} may be converted into sulfite-sulfur. The leaves were incubated in a medium containing S^{35}-labeled sulfate and $3\times10^{-2}M$ non-labeled sulfite, and the radioactivity of sulfite-S^{35} was measured. The results are shown in Table II. Radioactivity

TABLE II

Reduction of Sulfate to Sulfite in Excised Leaves

Experiment number	Total radioactivity of absorbed S^{35} in the leaves infiltrated with S^{35}-labeled sulfate and non-labeled sulfite	Intensity of incorporation of S^{35} into organic compounds in the leaves infiltrated with only S^{35}-labeled sulfate	Radioactivity of sulfite in the leaves infiltrated with S^{35}-labeled sulfate and non-labeled sulfite	Radioactivity of sulfite in the leaves infiltrated only with S^{35}-labeled sulfate
	c.p.m.	%	c.p.m.	c.p.m.
1	14,000	7	57	0
2	80,000	3	30	0
3	75,000	3	70	0
4	43,000	3	59	—

was not detected in the sulfite fraction in the case of incubation with S^{35}-labeled sulfate only, however, a slight conversion of sulfate-S^{35} into sulfite-S^{35} occurred when S^{35}-labeled sulfate was infiltrated together with non-labeled sulfite into the excised leaves. Thus, it is conceivable that sulfate reductase system may be operating in the plant tissue.

Incorporation of Sulfite-sulfur into Organic Compounds—If sulfite is an intermediate in the synthetic pathway from sulfate to sulfur-containing amino acids, sulfite-S^{35} may be incorporated into sulfur-containing amino acids. Excised leaves of mung bean were incubated in a solution containing S^{35}-labeled sulfite, and the intensity of incorporation of S^{35} into the compounds other than sulfate and sulfite was determined. As shown in Table III, the

results indicate that sulfite-sulfur was incorporated into the compounds other than sulfate and sulfite in both acid-soluble and acid-insoluble fractions,

TABLE III

Conversion of Sulfite-Sulfur into Organic Forms

Duration of incubation	Infiltrated[1] substances	Distribution of isotope[2]		
		acid-soluble fraction		acid-insoluble fraction
		organic S^{35},[3]	sulfate-S^{35}	
hr.		%	%	%
3	$Na_2S^{35}O_4$	0	99	1
3	$Na_2S^{35}O_3$	23	51	26
17	$Na_2S^{35}O_4$	0	98	2
17	$Na_2S^{35}O_3$	9	50	41
21[4]	$Na_2S^{35}O_4$	0	87	13
21[4]	$Na_2S^{35}O_3$	0	40	60

1) S^{35}-labeled sulfate did not contain the carrier but S^{35}-labeled sulfite contained non-labeled sulfite at the concentration of $5\times10^{-3}M$.

2) The values show the ratio of the radioactivity of S^{35} in each fraction in percentage of total S^{35} absorbed.

3) This fraction was the supernatant after precipitating out both sulfate and sulfite by adding $BaCl_2$ after treating with I_2.

4) Streptomycin was added in a sufficient amount to inhibit the growth of microorganisms.

and further that the rate of incorporation of sulfite-sulfur was higher than that of sulfate-sulfur. Table IV shows the effect of non-labeled sulfate and

TABLE IV

Effect of Non-labeled Sulfate and Sulfite on the Conversion of Sulfite-S^{35} into Organic Forms

Infiltrated substances	Amount of S^{35} in the compounds other than sulfate and sulfite in percentage of total S^{35} absorbed (%)
S^{35}-labeled sulfate	9
S^{35}-labeled sulfite	41
S^{35}-labeled sulfite+non-labeled sulfate	44
S^{35}-labeled sulfite+non-labeled sulfite	33

sulfite on the conversion of sulfite-S^{35} into organic form. The rapid metabolism of sulfite was not interfered by the addition of non-labeled sulfate at

a concentration of $4\times10^{-2}M$. The same concentration of non-labeled sulfite lowered the rate of incorporation of sulfite-S^{35} into the compounds other than sulfate and sulfite. Thus, it is evident that sulfite is rapidly metabolized into some organic compounds without back oxidation to sulfate.

Identification of Labeled Compounds Synthesized from Labeled Sulfite—The nature of the labeled compounds synthesized from S^{35}-labeled sulfite in excised leaves was investigated. Plants were incubated in a solution containing S^{35}-labeled sulfite and non-labeled sulfite at the concentration of $2\times10^{-3}M$. Results of two dimentional paper chromatography of the labeled compounds of the acid-soluble fraction are shown in Fig. 1. It was found that cystine

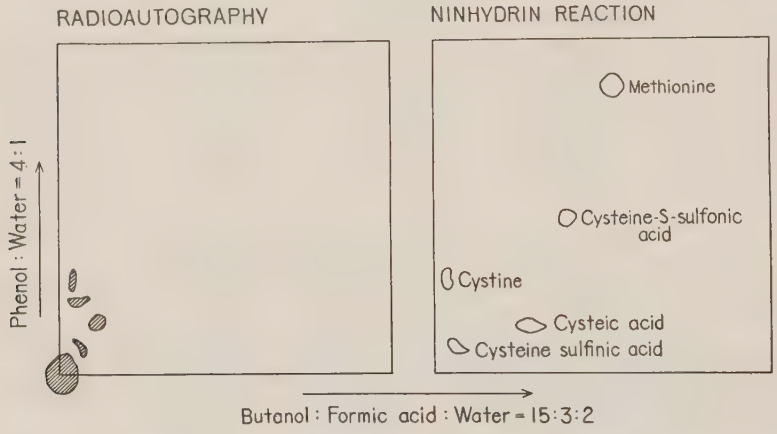

Fig. 1. Paperchromatography of labeled compounds.

and cysteine-sulfinic acid were radioactive and that there exists other unknown labeled compounds. However, it is certain that neither methionine nor cysteine-S-sulfonic acid were radioactive.

Labeled compounds in acid-soluble and acid-insoluble fractions were studied by Amberlite IRA 400 column chromatography (*13*). Fig. 2A shows the elution curve of the reference substances such as cystine and cysteic acid determined by colorimetric analysis with ninhydrin (*14*). The elution chromatography of cystine and unknown labeled compounds in both fractions is shown in Fig. 2B. It clearly shows the presence of radioactive cystine and unidentified compounds other than cystine and cysteic acid.

These experiments indicate that cystine, cysteine-sulfinic acid and some unidentified substances were synthesized from sulfite by the excised leaves.

Content of Labeled Amino Acids in Incubated Leaves—Determination was made of the percentage of absorbed S^{35} incorporated into sulfur-containing amino acids. Two hundred mg. of L-cystine were added to the acid-soluble and acid-insoluble fractions as a carrier compounds. The mixture was then treated with iodine solution, and cystine was crystallized repeatedly until the specific radioactivity did not decrease (*15*). The final crystals of cystine were decomposed to sulfate by the method described by Pirie (*12*), and

the specific radiactivity of $BaSO_4$ prepared by the addition of $BaCl_2$ was determined. Radioactivity of methionine-sulfur was also determined in a

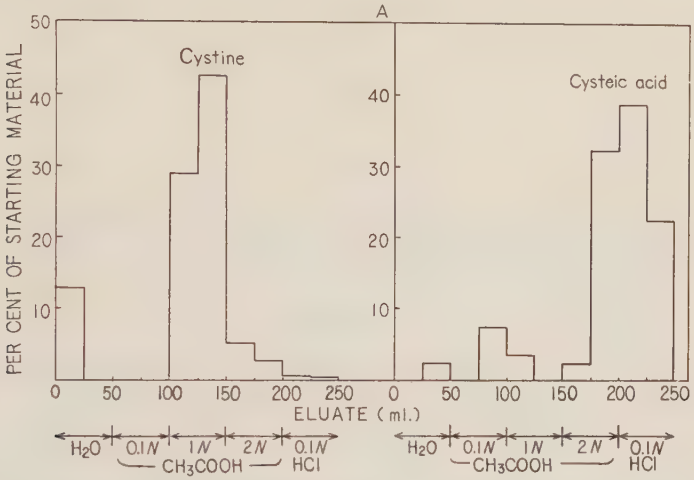

FIG. 2A. Column chromatography of cystine and cysteic acid.

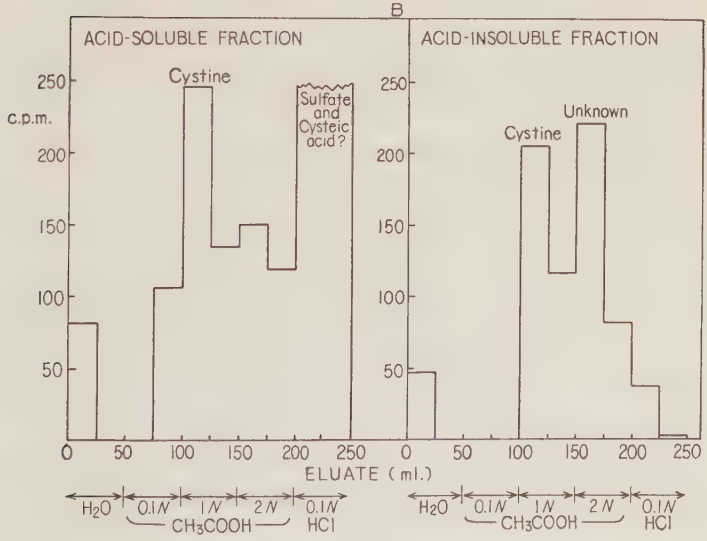

FIG. 2B. Column chromatography of labeled compounds.

similar way, after repeated crystallization by Takayama and Tsuchiya's method (*16*). The results are shown in Table V. Cystine and methionine were found to be labeled in both fractions, although labeled methionine was not detected in the acid-soluble fractioh by the paper chromatographic method.

TABLE V

Rate of Incorporation of Sulfite-Sulfur into Sulfur-Containing Amino Acids

	Acid-soluble fraction (%)[1]	Acid-insoluble fraction (%)[1]
Sulfate-S^{35}	72	16
Cystine-S^{35}	4.8	21
Methionine-S^{35}	8.2	24

1) Amount of S^{35} incorporated in the sulfur-containing amino acids in percentage of total absorbed S^{35}.

DISCUSSION

The observed inhibition of the incorporation of sulfate-sulfur into organic compounds by the simultaneous infiltration of sulfite seems not to be due to oxidation of the sulfite and subsequent dilution of the specific radioactivity of S^{35}-labeled sulfate. Rather it might have occurred either by the preferential utilization of sulfite for the synthesis of sulfur-containing amino acids or by the inhibitory effect of sulfite on the conversion of sulfate-sulfur into organic form. However, since the reduction of sulfate to sulfite was not inhibited and since there was a rapid incorporation of sulfite-sulfur into organic compounds, the second possibility seems to be excluded. It seems that sulfite is utilized preferentially for the formation of sulfur-containing organic compounds.

It is important that sulfate-S^{35} was found to be converted into sulfite-S^{35}, if the excised leaves of mung bean were incubated in the presence of S^{35}-labeled sulfate and non-labeled sulfite, because this fact indicates the presence of sulfate reductase in this plant. The same results were reported recently using tobacco leaves by Fromageot and Perez-Milan (*17*). Wilson and Bandurski (*18*), Ishimoto (*19*) and Peck (*20*) found that cell free extracts of some microorganisms were capable of reducing sulfate to sulfite in the presence of adenosine triphosphate. All those results suggest that the first step in the formation of sulfur-containing organic compounds utilizing sulfate is the reduction of sulfate to sulfite.

The hypothesis that sulfite may be an intermediate in the pathway of formation of sulfur-containing organic compounds from sulfate is also supported by the fact that sulfite-S^{35} is readily incorporated into sulfur-containing amino acids and some other organic compounds.

Thus, all the results are compatible with the hypothesis that sulfite is an intermediate in the synthetic pathway of sulfur-containing organic compounds from sulfate. However, the detection of labeled cysteine-sulfinic acid and unidentified other sulfur-containing substances might indicate another possibility. Thus, the above experiments cannot exclude the possibility that sulfate is converted to cysteine-sulfinic acid or unidentified substances

without passing through sulfite, providing the interconversion between the intermediate (*) and sulfite are very rapid as shown by the following scheme.

$$SO_4^{--} \longrightarrow * \longrightarrow \text{cysteine}$$
$$\Downarrow\uparrow$$
$$SO_3^{--}$$

SUMMARY

It has been observed that the simultaneous infiltration of S^{35}-labeled sulfate and non-labeled sulfite into the excised mung bean leaves, which were able to convert sulfate-sulfur into organic form, resulted in inhibition of the formation of S^{35}-labeled organic compounds. This inhibitery effect of sulfite on the metabolism of sulfate sulfur may be explained on the basis of the preferential utilization of sulfite as a source of sulfur for the synthesis of sulfur-containing organic compounds. It was also observed that sulfate-S^{35} was converted to sulfite-S^{35}. Futhermore, sulfite-S^{35} was readily converted to sulfur-containing amino acids such as cystine, methionine, cysteine-sulfinic acid, and some unidentified substances. These results support the hypothesis that sulfite is an intermediate in the biosynthetic pathway of sulfur-containing amino acids from sulfate.

The authors are indebted to Prof. I. Uritani in Laboratory of Biochemistry for his kind discussions during this investigation, and thanks are also due to Miss N. Harada in Laboratory of Food and Nutritional Chemistry of this Department for her partial participation in this work.

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METABOLISM OF L-ALLOISOCITRIC ACID IN ACHROMOBACTER*

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Of the four stereoisomers of isocitric acid, only L_S-isocitric acid has been known to be biologically active, as an intermediate of the tricarboxylic acid cycle and the other pathways. It has been found in this laboratory, however, that a number of *Penicillium* molds accumulate a large amount of L_S-alloisocitric acid, a diastereoisomer of isocitric acid, from glucose as a sole source of carbon (*1*, *2*). During the course of the studies on this new acid fermentation, it was necessary to estimate microquantities of L_S-alloisocitric acid. In order to establish an enzymatic determination method of the acid, a sutdy on the alloisocitrate decomposing bacteria was initiated. Besides such a technical purpose, the metabolism of alloisocitric acid itself, which has been believed to be unnatural, is of some interest. An adaptive enzyme, which was designated as alloisocitric dehydrogenase, was found from a newly isolated bacterium belonging to the genus *Achromobacter*, and was used for the enzymatic determination of the acid.

From the isolated bacterium, α-hydroxyglutaric dehydrogenase, playing a part in the anaerobic dismulation of alloisocitrate, was also found. Though α-hydroxyglutaric acid has been reported as a product of the anaerobic decomposition of citrate in animal tissues (*3*) and it has also been assumed as an intermediate metabolite in the pathway from lysine to α-ketoglutarate (*4*), an enzyme responsible for its formation and decomposition has never been reported. Our survey suggested that the enzyme was contained constitutively in many bacteria.

Experimental results on these two enzymes are described in this paper. A preliminary account on this work has been published elsewhere (*2*).

EXPERIMENTALS

Isolation and Cultivation—A medium for the isolation of the decomposing bacterium contained 0.8 g. of NH_4NO_3, 0.1 g. of KH_2PO_4, 0.1 g. of $MgSO_4 \cdot 7H_2O$ and a trace of $FeSO_4$ in 100 ml. of tap water. The isolated bacterium was grown aerobically by shaking cultivation at 30° in a bouillon-alloisocitrate medium containing 1 g. of meat

* The following abbreviations are used in this paper: DPN and TPN, di- and tri-phosphopyridine nucleotides; DPNH, reduced diphosphopyrindine nucleotide; AMP, adenosine monophosphate; Tris, tris(hydroxymethyl)-aminomethame;

extract, 1 g. of pepton, 1.5 g. of NaCl and 0.6 g. of trisodium alloisocitrate in 100 ml. of tap water.

Assay of the enzymes—Conventional Warburg apparatus and manometric techniques were employed for measuring the oxygen consumption. Reduction of pyridine nucleotides was measured spectrophotometrically at 340 mμ. One unit of alloisocitric or α-hydroxyglutaric dehydrogenases is defined as that amount which causes an initial rate of increase or decrease in optical density at 340 mμ of 0.001 per minute under the condition described as follows: a cell (light path = 1 cm) contained 280 μM of Tris buffer, pH 7.4, 10 μM of alloisocitrate or α-ketoglutarate, 1.0 μM of DPN or 0.1 μM of DPNH, and 0.1 ml. of enzyme preparation in a final volume of 3.0 ml. The reaction was initiated with the addition of the substrate. For the determination of the Michaelis constant of alloisocitric dehydrogenase, the reaction was measured spectrophotometrically under the same conditions as mentioned above.

Analytical Methods—Estimation of α-ketoglutaric acid was done according to the metod of Friedemann and Haugen (*5*). Radioactivities in carbon dioxide were counted with a conventional G-M counter after conversion to $BaCO_3$ and corrected for the self absorption. Optical rotation of the molybdate complex of α-hydroxyglutaric acid was measured according to the method of Krebs and Eggleston (*6*).

Paperchromatography—Paperchromatography of organic acids was carried out with Toyo No. 50 filter paper in a solvent mixture consisting of butanol, formic acid, and water (4 : 1.5 : 1). 2.4-Dinitrophenylhydrazone of α-ketoglutaric acid was chromatographed with filter paper using butanol saturated with 3 per cent aqueous ammonium as solvent.

Chemicals—Calcium L-alloisocitrate was a fermentation product of *Penicillium purpurogenum var. rubrisclerotium* No. 1148 prepared at the Kyowa Fermentation Industry Co. Trisodium alloisocitrate was prepared from the calcium salt. 1,6-C^{14}-Alloisocitric acid was obtained by the fixantion of $C^{14}O_2$ during the study on the alloisocitric acid fermentation by the *Penicillium* mold*. DL-Hydroxyglutaric acid was a gift from the Ajinomoto Co. Di- and tri-phosphopyridine nucleotides and DL-isocitric acid were purchased from the Mann Research Laboratories Co. Reduced diphosphopyridine nucleotide was prepared enzymatically using alcohol dehydrogenase (*7*).

RESULTS

Isolation and Identification of Alloisocitrate Decomposing Bacterium—Bacteria able to grow on the medium containing L-alloisocitrate as a sole carbon source were isolated from soil and water. One of them, which was identified to belong to the genus *Achromobacter* according to Bergey's Determinative Bacteriology, was used throughout this study. Taxonomical descriptions were as follows.

Rods 1.4 to 1.6 by 0.6 microns. Motile by means of about 6 peritrichous flagella. Gramnegative.
Agar colonies: Ciruclar, convex, smooth.
Agar slant: Growth moderate, filiform, butyrous, smooth.
Nutrient broth: Turbid.
Gelatin stab: No liquefaction.
Litmus milk: Slightly alkaline.
No acid or gas from glucose, sucrose, lactose, glycerol, and xylose.

* unpublished data to be reported elsewhere.

Methyl red test, negative.
Acetylmethylcarbinol not produced
Nitrites produced from nitrates.
Aerobic, facultative. Poor growth at 38°. No pigment formed.

Though this bacterium had a weak activity of oxidizing alloisocitrate when it grew in ordinary bouillon media (Q_{O_2}, 10′; 0.5 μl. per mg. dried cells), the activity was remarkably strengthened when they were allowed to grow in the presence of alloisocitate. Adaptive formation of this enzyme activity was greater in the bouillon-alloisocitrate medium than in the synthetic medium containing the acid as a sole carbon source. On the other hand, the bacterium had a strong activity of oxidyzing α-hydroxyglutarate even when alloisocitrate was omitted from the growing medium.

Preparation of the Cell-Free Extracts—The *Achromobacter* was grown aerobically by shaking cultivation in the bouillon-alloisocitrate medium for about 20 hours at 30°. The organism was harvested by centrifugation and washed twice with distilled water. The washed cell suspension oxidized L$_s$-alloisocitrate completely (4.5 M of oxygen uptake per 1 M of the acid, Q_{O_2}, 10′; 12.0 μl. per mg. dried cell) and also oxidized α-hydroxyglutarate several times faster than they did with allo-isocitrate. The harvested bacterial paste was lyophilized. About 5 g. of lyophilized bacteria was obtained from 3 liters of the cultures. Five grams of lyophilized bacterial powder were ground in a mortar at 0° with 5 g. of alumina and 40 ml. of 0.1 *M* Tris buffer, pH 7.4. After centrifugation of the mixture at 15,000×*g* for 20 minutes in the cold, a crude cell-free extract containing 500-600 units of alloisocitric dehydrogenase per ml. was obtained*. This activity did not sediment after spinning at 100,000×*g* for 1 hour. The extract could be stored for several weeks at −20° with little loss of activity.

DPN-Specific L-Alloisocitric Dehydrogenase—Active oxygen uptake and CO_2 evolution occurred when the extract was incubated with L-alloisocitrate in the presence of DPN and methylene blue, and a ketonic compound appeared in the incubation mixture. It was identified as α-ketoglutaric acid by paperchromatography of the 2,4-dinitrophenylhydrazone. Absorption maximum of the hydrazone obtained was 380 mμ, which was also in good coincidence with that of α-ketoglutaric acid. Approximate stoichiometry between the oxygen uptake, and the production of CO_2 and α-ketoglutarate was observed as shown in Table I.

Oxidation of L-alloisocitrate was found to be dependent upon DPN, but not upon TPN. Increase of optical density at 340 mμ with L-alloisocitrate was observed only when DPN was added (Fig. 1). Though L-isocitric dehydrogenase was proved in the extract, it was specific for TPN but not for DPN. Therefore, it is certain that the alloisocitrate oxidation in the

* In the presence of DPN and methylene blue, the cell-free extract oxidized L-alloisocitarate far more rapidly than α-hydroxyglutarate (under the condition simialar to that described in Fig. 4, Q_{O_2}, 10, for L-alloisociatrate was 22.1 μl., while that for α-hydroxyglutarate was 2.1 μl.).

extract occurred independently of the TPN-isocitric dehydrogenase.

TABLE I

Stoichiometry of L-*Alloisocitrate Oxidation*

In a final volume of 2.0 ml. in the Warburg vessels, the following components were incubated at 30° for 140 minutes; 180 μM of Tris buffer, pH 7.9, 5 μM of L-alloisocitrate, 0.1 μM of DPN, 1 μM of methylene blue, and 0.5 ml of the crude extract. Gas phase, air.

L-Alloisocitrate added	O_2 uptake	α-Ketoglutarate formed	CO_2 formed
μM	μM	μM	μM
5.0	2.36	5.30	4.62

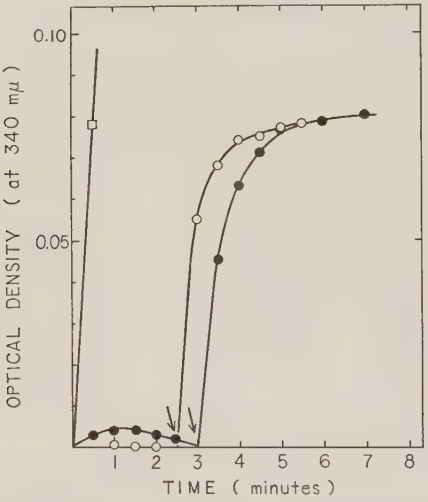

FIG. 1. Reduction of DPN with L-alloisocitrate.

Incubation mixtures contained in a final volume of 3.0 ml., 280 μM of Tris buffer, pH 7.4, and 0.1 ml. of the crude enzyme with the following additions: —○—; 10 μM of L-alloisocitrate and 1.0 μM of TPN. At the time indicated by the arrow, 1.0 μM of DPN was added. —●—; 1.0 μM of DPN and 10 μM of DL-isocitrate. At the time indicated by the arrow, 1.0 μM of L-alloisocitrate was added. —□—; 10 μM of DL-isocitrate and 1.0 μM of TPN.

The addition of EDTA (final conc., $3.3\times10^{-3}\,M$) completely inhibited isocitric dehydrogenase without inhibition of alloisocitric dehydrogenase activity. The latter activity did not decrease at all even after dialyzing 3.0 ml. of the crude extract against 1.5 liters of the solution of EDTA

(10^{-3} *M*) with stirring for 16 hours in the cold. Reactivation by adenosinemonophosphate, reported with DPN-specific isocitric dehydrogenase from yeast and mammalian tissues, was not observed.

Reversibility of the reaction catalyzed by alloisocitric dehydrogenase was studied using $C^{14}O_2$ with a partially purified enzyme preparation. The enzyme used was fraction F-3 described in the following part of this paper. The incubation mixture placed in a Warburg vessel contained 1.0 ml. of the enzyme, 20 μM of $NaHC^{14}O_3$ (5×10^5 c.p.m.), 30 μM of L-alloisocitrate, 10 μM of α-ketoglutarate, 0.2 μM of DPN, and 300 μM of phosphate buffer, pH 7.4 in a total volum of 5.2 ml. in a Warburg vessel. After 4 hours of incubation at 30°, hydrochloric acid was tipped in order to stop the reaction and to remove residual $NaHC^{14}O_3$, and an aliquot of the solution was evaporated to dryness and its radioactivity was counted directly. Any significant incorporation of radioactivity into alloisocitrate was not observed (even the highest incorporation observed was less than 0.05 per cent of the radioactivity added).

The Michaelis constant of the enzyme for L-alloisocitrate was calculated to be approximately 2.5×10^{-4} *M*. The optimum pH of the enzyme was found to be between 7 and 8.

Presence of α-Hydroxyglutaric Dehydrogenase in the Crude Extracts—Though the oxidation of alloisocitrate by the crude extract was very rapid in the initial stage, the rate of the oxidation decreased considerably when the amount of O_2-uptake attained about the half of the theoretical final value and it took very long time till the oxygen uptake reached the theoretical value (Fig. 7). The incomplete oxidation was more distinct when the reaction was traced by measuring the reduction of DPN spectrophotometrically at 340 mμ. As shown in Fig. 1, the increase in optical density at 340 mμ came to a halt at about 0.1 even when 1 μM of DPN (the amount expected to give an optical density of 2.1 at 340 mμ when the reduction was completed) and a large excess of L-alloisocitrate were present in the incubation mixture. The maximum level of the reduction of DPN was lowered down on the addition of α-ketoglutarate but improved on the addition of semicarbazide (Fig. 2). Since the enzyme preparation failed to catalyze the reductive carboxylation of α-ketoglutarate as described previously, these observations could not be explained by the reversibility of the reaction. Finding of the rapid reduction of α-ketoglutarate by α-hydroxyglutaric dehydrogenase in the crude extract clearly elucidated these results.

The addition of α-ketoglutarate to the incubation mixture containing the extract and DPNH caused an apparent decrease of optical density at 340 mμ (Fig. 3). α-Ketoglutarate was reduced with DPNH to α-hydroxyglutarate which was identified by paperchromatography. The crude extract (0.5 ml.) was incubated for 4 hours at 30° with 50 μM of α-ketoglutarate, 10 μM of DPNH, 300 μM of Tris buffer, pH 7.4, in a total volume of 5.0 ml., in a Thunberg tube, gas phase of which was exchanged with N_2. After the reaction was stopped, residual α-ketoglutarate was removed by conversion to the

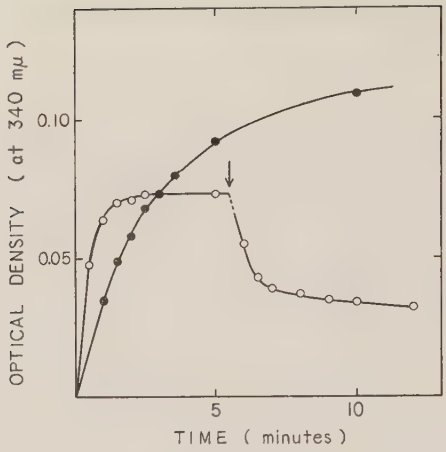

FIG. 2. Effect of semicarbazide and α-ketoglutarate on alloisocitric dehydrogenase.

Incubation mixtures contained in a final volume of 3.0 ml., 280 μM of Tris buffer, pH 7.4, 10 μM of L-alloisocitrate, 1.0 μM of DPN, and 0.1 ml. of the crude extract. —○—; Without semicarbazide. At the time indicated by the arrow, 20 μM of α-ketogblutarate was added. —●—; With 80 μM of semicarbazide (final conc., $2.67 \times 10^{-2} M$).

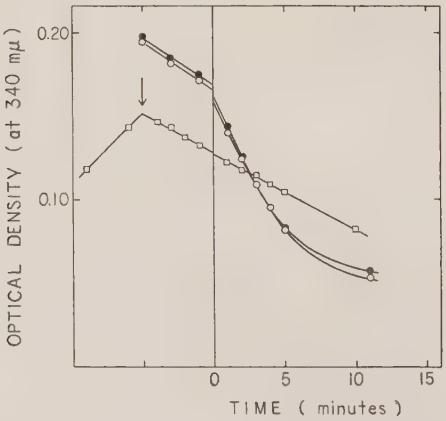

FIG. 3. Reduction of α-ketoglutarate with DPNH.

Incubation mixtures contained, in a final volume of 3.0 ml., 280 μM of Tris buffer, pH 7.4, 0.1 ml. of the crude extract, with the following additions: 10 μM of α-ketoglutarate was added at time 0. —○—; 0.1 μM of DPNH. —●—; 0.1 μM of DPNH and 10 μM of EDTA (final conc., $3.3 \times 10^{-3} M$). —□—; 1.0 μM of TPN and 10 μM of L-malate. At the time indicated by the arrow, 10 μM of EDTA was added.

2,4-dinitrophenylhydrazone and subsequent extraction of the hydrazone with ethylacetate. The residual solution was acidified by sulfuric acid and extracted continuously with ether for 24 hours, and the extract was applied to paperchromatography. Both the authentic α-hydroxyglutaric acid and the sample obtained gave two identical spots, one being that of the free acid (R_f 0.61) and the other that of the γ-lactone (R_f 0.76). Though the configuration of α-hydroxyglutaric acid formed was not determined directly, the molybdate complex of the acid was found to be levorotatory. This indicates that the acid was (−)-α-hydroxyglutaric acid (*6*).

Under an anaerobic condition, alloisocitric dehydrogenase in the crude extract linked with this α-ketoglutarate reducing activity by mediation of DPN, and dismutation of alloisocitrate to α-hydroxyglutarate and CO_2 as shown below occured. This anaerobic decarboxylation activity of alloisocitrate disappeared after dialysis of 4.0 ml. of the crude extract against 2 liters of distilled water with stirring for 17 hours in the cold, but the addition of DPN restored this activity (Table II).

(a) L-Alloisocitrate + DPN $\longrightarrow$ α-Ketoglutarate + CO_2 + DPNH
(b) α-Ketoglutarate + DPNH $\rightleftarrows$ α-Hydroxyglutarate + DPN

Overall: L-Alloisocitrate $\longrightarrow$ α-Hydroxyglutarate + CO_2

Table II

Effect of DPN on the Anaerobic Decarboxylation of L-Alloisocitrate

In a final volume of 2.0 ml. in the Warburg vessels, the following components were incubated at 30° for 40 minutes; 120 μM of Tris buffer, pH 7.4, 5 μM of L-alloisocitrate, each 3 μM of Mn^{++} and Mg^{++}, and 0.5 ml. of the enzyme preparation with or without 0.1 μM of DPN. Gas phase, N_2

	Addition of DPN	CO_2 evolved
Crude extract	+	49.3 μl.
Dialyzed prep.	+	32.8
	−	4.3

The disproportion between the O_2 uptake and CO_2 evolution during the course of oxidation of alloisocitrate was also found using 1,6-C^{14}-alloisocitrate as the substrate. Evolution of $C^{14}O_2$ from labeled alloisocitrate reached rapidly the theoretical level, whereas the oxygen uptake proceeded only slowly and ceased below the theoretical value (Fig. 4). This indicated that the dismutation of alloisocitrate to α-hydroxyglutarate occurred simulataneoulsly even under aerobic conditions.

Though the reaction was very much in favor of reduction of α-ketoglutarate, the oxidation of α-hydroxyglutarate by the enzyme could be observed clearly. The enzyme catalyzing the latter reaction may be designated as α-hydroxyglutaric dehydrogenase. When DPN and a large amount of α-

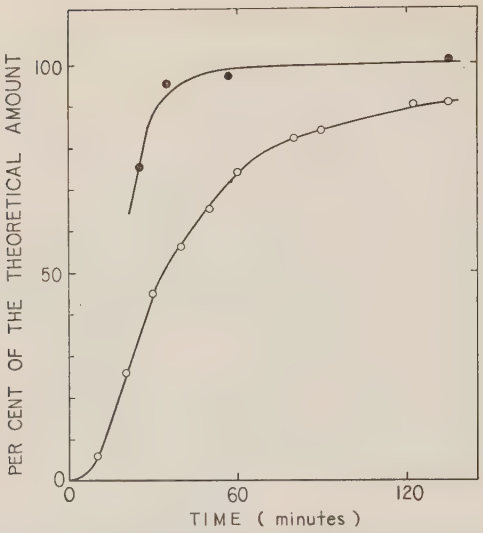

FIG. 4. Disproportion between O_2 uptake and CO_2 evolution during the oxidation of L-alloisocitrate.

Incubation mixtures placed in the Warburg vessels contained in a final volume of 2.0 ml., 170 μM of phosphate buffer, pH 7.4, 0.1 μM of DPN, 1.0 μM of methylene blue, 0.5 ml. of the crude extract, and 6.55 μM of 1,6-C^{14}-alloisocitric acid (total radioactivity, 14,900 c.p.m.). Gas phase, air. Temperature, 30°. —○—; Oxygen uptake. —●—; Radioactivity in carbon dioxide evolved.

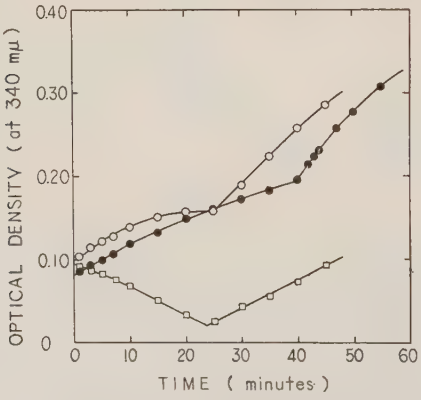

FIG. 5. Reduction of DPN with α-hydroxyglutarate.

Incubation mixtures contained in a final volume of 3.0 ml., 180 μM of phosphate buffer, pH 7.4, 2.0 μM of DPN, and 1.5 ml. of the crude extract, with the following additions: —○—; 100 μM of DL-α-hydroxyglutarate. —●—; 100 μM of DL-α-hydroxyglutarate and 500 μM of semicarbazide. —□—; 10 μM of α-ketoglutarate.

hydroxyglutarate were added to the crude extract (see Fig. 5), the optica density at 340 mμ increased slowly, and the process was soon leveled off at a stage of very low equilibrium value; later, however, it resumed to increase markedly, indicating the occurrence of reduction of DPN coupled with the dehydrogenation of α-ketoglutarate which had been formed from α-hydroxyglutarate. By the addinion of a large amount of semicarbazide to the reaction system, the low equlibrium point of the first step was removed. A prompt reduction of DPN after a lag period was observed when α-ketoglutarate was used as substrate.

Partial sepaation of α-hydroxyglutaric dehydrogenase and alloisocitric dehydrogenase was carried out by fractional precipitation with ammonium sulfate. The first fractionation of the crude extract (17.0 ml. in volume) was carried out at salt concentrations of 40 and 60 per cent saturation at 3°, and each precipitate was dissolved in 5.0 ml. of Tris buffer, 0.1 *M*, pH 7.4. They were designated as F-1 and F-2 respectively. The second fractionation at 40 per cent saturation of the salt from F-1 was carried out and the precipitate was dissolved in the same amount of buffer (F-3). The ratios of alloisocitric dehydrogenase activity to α-hydroxyglutaric dehydrogenase activity were 0.31 in the crude extract, 0.94 in F-1, 0.10 in F-2, and 2.0 in F-3. Maximum value of reduction of DPN with alloisocitrate was greatly improved as the ratio increased (Fig. 6).

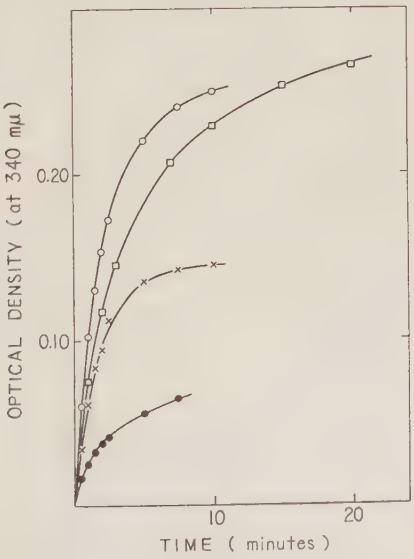

FIG. 6. Effect of fractionation with ammonium sulfate on reduction of DPN with L-alloisocitrate.

Incubation mixtures contained in a final volume of 3.0 ml., 280 μM of Tris buffer, pH 7.4, 10 μM of L-alloisocitrate, 1.0 μM of DPN, and 0.1 ml. of the enzyme preparations described in the text. —×—; The crude extract. —○—; F-1 —●—; F-2 —□—; F-3.

α-Hydroxyglutaric dehydrogenase was not inhibited by the adddition of EDTA (final conc., $3\times10^{-3}M$), but dialyzing 3.0 ml. of the crude extract against 1.5 liters of the solution of EDTA ($10^{-3}M$) for 16 hours with stirring in the cold caused inhibition by 63 per cent. The addition of Mn^{++} or Mg^{++} (final conc., $10^{-4}M$) recovered this inhibition.

The enzyme was specific to DPN but not to TPN as shown in Fig. 3. In this experiment, reduced TPN was formed by TPN-specific malic enzyme contained in the extract with the addition of malate. Before the incubation with α-ketoglutarate, malic enzyme activity was removed by the addition of EDTA without any effeet on α-hydroxyglutaric dehydrogenase. The crude extract had neither lactic nor glycolic dehydrogenase activities and the ratio of DPN-malic dehydrogenase activity to α-hydroxyglutaric dehydrogenase activity differed between the crude extract and the dialyzate against EDTA. These facts suggested that the enzyme was distinct from the other α-hydroxy acid dehydrogenases.

The pH optimum of the enzyme was approximately 7.0.

Distribution of α-hydroxyglutaric dehydrogenase—Among the bacteria tested, all *Pseudomonads* constitutively had the activity of oxidizing α-hydroxyglutarate. They were *Ps. aeruginosa* B-11-2, *Ps. fluorescens* B-5-6, and *Ps.*

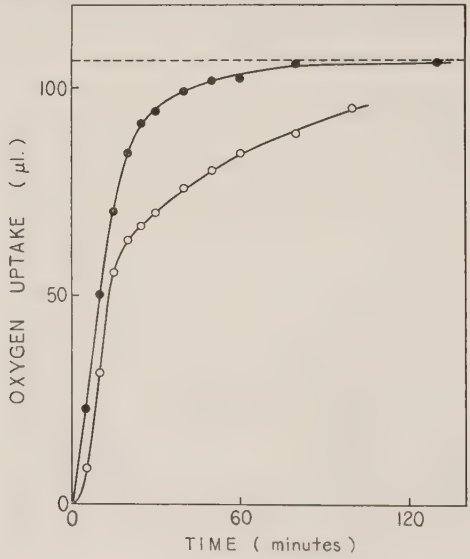

Fig. 7. Oxidation of L-alloisocitrate.

Incubation mixtures placed in the Warburg vessels contained in a final volume of 2.0 ml., 170 μM of phosphate buffer, pH 7.4, 9.4 μM of L-alloisocitrate, 0.1 μM of DPN, 1.0 μM of methylene blue, and 1.0 ml. of the crude extract. Gas phase, air. Temperature, 30°. —○—; Without semicarbazide. —●—; With 250 μM of semicarbazide (final conc., $2.5\times10^{-1}M$).

dacunhae B-1-3. Though we could not find any other species oxidizing

the acid from the collection of our laboratory, this activity was very common in the alloisocitrate decomposing bacteria which we isolated from various sources.

The bacteria constitutively having apparent alloisocitric dehydrogenase activity could not be found.

Determination of L-Alloisocitrate—On the basis of the results described above, a manometric determination method of alloisocitrate using the crude extracts was devised. Though the crude extracts contained other dehydrogenases such as TPN-isocitric and DPN-malic dehydrogenases and TPN-malic enzyme, these were inhibited effectively by the addition of EDTA (3×10^{-3}M) without inhibition of alloisocitric dehydrogenase. Theoretical oxygen uptake could be achieved by the addition of relatively large amounts of semicarbazide (final conc., $2.5\times10^{-1}M$) (Fig. 7). The amount of L-alloisocitrate calcurated from the oxygen uptake agreed well with that calculated by the method based on the optical rotation (*2*).

DISCUSSION

The analogy between L-alloisoctric dehydrogenase and L-isocitric dehydrogenase is not absolute, although both these enzymes bring about the oxidation and decarboxylation of their substrates to α-ketoglutaric acid. Unlike TPN-specific L-isocitric dehydrogenase, L-alloisocitric dehydrogenase showed no dependence on metal even after the dialysis against EDTA. Irreversibility of the reaction catalyzed by L-alloisocitric dehydrogenase is similar to that of DPN-specific L-isocitric dehydrogenase from mammalian tissues and yeast. It was reported with the DPN-isocitric dehydrogenase that oxalosuccinic acid did not appear to be an intermediate. The experiment concerning this point have not yet been carried out with alloisocitric dehydrogenase. Because L-alloisocitric acid has a reversed configuration of β-carbon atom compared to that of L-isocitric acid, intermediate oxalosuccinic acid of L-alloisocitric dehydrogenase, if present, may be an enantiomorph of that in L-isocitric dehydrogenease. Requirement of AMP reported for DPN-isocitric dehydrogenase was not observed with this enzyme.

Enzymatic reduction of many α-keto acids to corresponding α-hydroxy acids had been observed commonly in many organisms but the enzyme responsible for the reduction of α-ketoglutaric acid has remained unnoticed. The fact that α-hydroxyglutaric dehydrogenase seems to distribute widely among the bacteria indicates that this enzyme may operate as a DPNH-acceptor system *in vivo* like alcohol dehydrogenase or lactic dehydrogenase.

SUMMARY

1. A strain of *Achromobacter* having a capacity for decomposing L_S-alloisocitrate, which had been believed to be an unnatural diastereoisomer of isocitric acid, was isolated.

2. From this organism an adaptive enzyme, DPN-specific L-alloisocitric dehydrogenase, which oxidizes the acid to α-ketoglutaric acid, was extracted. The reaction was found to be irreversible and seemed to require no metals nor other cofacters.

3. A constitutive enzyme, DPN-specific α-hydroxyglutaric dehydrogenase was for the first time found in this bacterium. Dependence of the enzyme on Mg^{++} or Mn^{++} was revealed only after the dialysis against EDTA.

4. Anaerobic dismutation of L-alloisocitric acid to α-hydroxyglutaric acid was proved in a crude cell-free extract of the organism.

5. The activity of oxidizing α-hydroxyglutaric acid was found to be widely distributed among different species of bacteria. It was discussed that the α-hydroxyglutaric dehydrogenase may take a role of DPNH-acceptor system *in vivo*.

6. A procedure was devised to estimate L-alloisocitric acid manometrically using the crude extract of the bacterium with EDTA and semicarbazide.

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STUDIES ON CYTOCHROME A*

VI. EFFECT OF ALDEHYDE REAGENTS ON CYTOCHROME A

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It has already been postulated in the classical works of Warburg and Fischer and Seemann (*1*) that there is a formyl side chain in the prosthetic group of the respiratory ferment. About twenty years later, the chemical structure of the prosthetic group of cytochrome oxidase was again studied by Rawlison *et al.* (*2*, *3*) and many results on spectral properties of porphyrin a were accumulated by the detailed investigations of Lemberg and his colleagues (*4*, *5*). Their studies and those of Dannenberg and Kiese (*6*) have established that the prosthetic group of cytochrome a was an iron porphyrin compound containing a formyl group.

According to the recent findings of Lemberg (*5*), porphyrin a differs from protoporphyrin in having formyl and ethylenic side groups and a long chain aliphatic group. The latter group would confer lipophilic characters on the prosthetic group. This is of particular interest because of the association of cytochrome a with lipids in the mitochondria. However, the participation of the formyl group in the functioning of cytochrome a is still unknown.

On the other hand, it has been demonstrated by several investigators (*7–10*), that transaminases and decarboxylases, which require pyridoxal phosphate as coenzyme, are strongly inhibited by aldehyde reagents. This indicates a possible binding of the reagents with the formyl group of the coenzyme. Furthermore, Okumura (*11*, *12*) and Masuda (*13*) have found that aldehyde reagents also have a strong inhibitory action on the activity of cyanide activated papain. The activity of some other enzymes are also influenced by these reagents (*14*). From these results, it may be assumed that the formyl group in the prosthetic group of cytochrome a plays an important role in the cytochrome oxidase reaction.

To study this, several investigations have been conducted on the effect of aldehyde reagents on cytochrome oxidase activity. The present paper

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deals with the inhibition of cytochrome oxidase by aldehyde reagents. Highly purified cytochromes a and c were used in an effort to elucidate the role of the formyl group of cytochrome a in the cytochrome oxidase reaction. The observed effect of aldehyde reagents on the spectral properties of cytochrome a, heme a and porphyrin a shows that some of the reagents may react with the formyl group but not with the heme iron. Furthermore, the two reagents, hydrazine and phenylhydrazine, can inhibit the cytochrome oxidase activity in a competitive way with cytochrome c. The results are also discussed from the point of view that the activity of cytochrome oxidase results in an interaction of cytochromes a and c in the presence of oxygen.

EXPERIMENTAL

MATERIALS AND METHODS

Highly purified cytochrome a was prepared by treating a heart muscle particle suspension with cholate and ammonium sulfate as described in the previous report (*15*). To determine the activity in a strongly activated state, Emasol 4130, a non-ionic detergent, was used as a solvent for the final preparation of cytochrome a, as reported previously (*16*).

Crystalline oxidized cytochrome c was prepared from beef heart by the method of Hagihara *et al.* (*17*). The crystals were dissolved in 0.1 *M* phosphate buffer, pH 7.4, and dialyzed against the same buffer for 4 hours before use.

Hemin a was isolated from the above purified preparation of cytochrome a by the acetone-HCl method as described previously (*15*). Porphyrin a was prepared from hemin a by the ferrous acetate-acetic acid method of Warburg and Negelein (*18*).

Crystalline catalase was prepared from beef liver by the method of Kitagawa and Shirakawa (*19*).

Emasol 4130, from Kao Soap Co., was used after dilution to 10 per cent (v/v) with 0.1 *M* phosphate buffer, pH 7.4.

Cytochrome oxidase activity was measured manometrically in a Warburg apparatus described previously (*16*).

The catalase activity was measured by the method of Bonnichsen *et al.* (*20*).

Absorption spectra were studied with a Cary spectrophotometer, Model 14, using cells of a 1 cm. light path.

RESULTS

Effect of Aldehyde Reagents on Cytochrome Oxidase—When measured manometrically, the cytochrome oxidase activity with purified cytochromes a and c was strongly inhibited by aldehyde reagents such as hydrazine, phenylhydrazine, hydroxylamine and sodium bisulfite. As can be seen in Table I, these reagents were effective inhibitors of cytochrome oxidase even at a concentration of $10^{-3}M$. There are no other strong inhibitors of cytochrome oxidase except cyanide. Other aldehyde reagents, such as semicarbazide and dimedone, showed no inhibition at a concentration of $10^{-2}M$.

This inhibition occurred immediately after the addition of the reagents.

and it was not influenced by preincubation of cytochrome a with the reagents. This means that over 90 per cent of the final inhibition was attained within 10 minutes after adding these reagents. Furthermore, the

TABLE I

Effect of Aldehyde Reagents on Cytochrome Oxidase Activity

Reagent	Concentration (M)	Inhibition (%)
Hydrazine	10^{-3}	100
	5×10^{-4}	72
	10^{-4}	0
Sodium Bisulfite	10^{-2}	75
	10^{-3}	50
	10^{-4}	15
Phenylhydrazine	10^{-3}	100
	5×10^{-4}	30
	10^{-4}	0
Hydroxylamine	10^{-3}	100
	5×10^{-4}	78
	10^{-4}	27
Semicarbazide	10^{-2}	0
Dimedone	10^{-2}	0

inhibition was reversible, as indicated by the following experiment. Cytochrome a in 0.1M phosphate buffer containing 0.5 per cent Emasol 4130 at pH 7.4 was incubated with $10^{-3}M$ of the aldehyde reagents for 60 minutes. Then the mixture was dialyzed against the same buffer for 20 hours to remove the reagents from the preparation. The preparation showed the same activity as in control experiments to which the reagents had not been added. On the basis of the above results it may be that the inhibition of the cytochrome oxidase reaction is due to the reaction between aldehyde reagents and the formyl group in the prosthetic group of cytochrome a. However, there is also a possibility that aldehyde reagents combine with the heme iron before they have a chance to react with the formyl group, because hydroxylamine, which was used as an aldehyde reagent, is a conventional heme iron binding inhibitor. It is necessary, therefore, to check the possibility that the reagents are able to react with heme iron. For its purpose, catalase was chosen as a hemoprotein without a formyl group. As shown in Table II, and as was reported by many workers, the catalase activity was completely inhibited by $10^{-4}M$ hydroxylamine. However, other reagents such as hydrazine, phenylhydrazine, and sodium bisulfite at a concentration of $10^{-3}-10^{-4}M$ had no inhibitory

effect as judged by assay of the catalase activity by the titration with permanganate. The effect of the reagents at a concentration of $10^{-3}M$ was also examined manometrically and it was difficult to observe a marked inhibition. Therefore, it seems likely that hydroxylamine is capable of inhibiting both the heme iron and the non-heme group of the enzyme, while the other aldehyde reagents have a greater affinity towards the formyl group than they do towards the heme iron. The point of attack by aldehyde reagents on cytochrome a will be discussed at the end of this paper.

TABLE II

Effect of Aldehyde Reagents on Catalase Activity

Reagent	Concentration (M)	Inhibition (%)
Hydrazine	10^{-3}	0
	10^{-4}	0
Sodium Bisulfite	10^{-3}	0
	10^{-4}	0
Phenylhydrazine[1]	10^{-4}	0
Hydroxylamine	10^{-4}	100

1) The reaction of catalase with $10^{-3}M$ phenylhydrazine was difficult to study because it was rapidly decomposed by titration with permanganate.

Nature of the Inhibition of Cytochrome Oxidase Activity—To clarify the mode of inhibition, the reaction was analyzed by the method of Lineweaver and Burk at two concentrations of aldehyde reagents and five concentrations of cytochrome c. The data were plotted by the double reciprocal procedure and the inhibition was found to be both competitive and non-competitive in type.

Fig. 1 shows that with respect to inhibition by phenylhydrazine and hydrazine all curves met at a common intercept when extrapolated to an infinite concentration of cytochrome c. Hence it follows that the inhibition is competitive. In contrast to the above reagents, it appears to be more nearly non-competitive than competitive with respect to the inhibition by hydroxylamine and sodium bisulfite.

It is most interesting that phenylhydrazine and hydrazine inhibit the cytochrome oxidase reaction in a competitive way with cytochrome c. This is the first report of such an inhibitor acting competitively with cytochrome c and specifically with cytochrome oxidase.

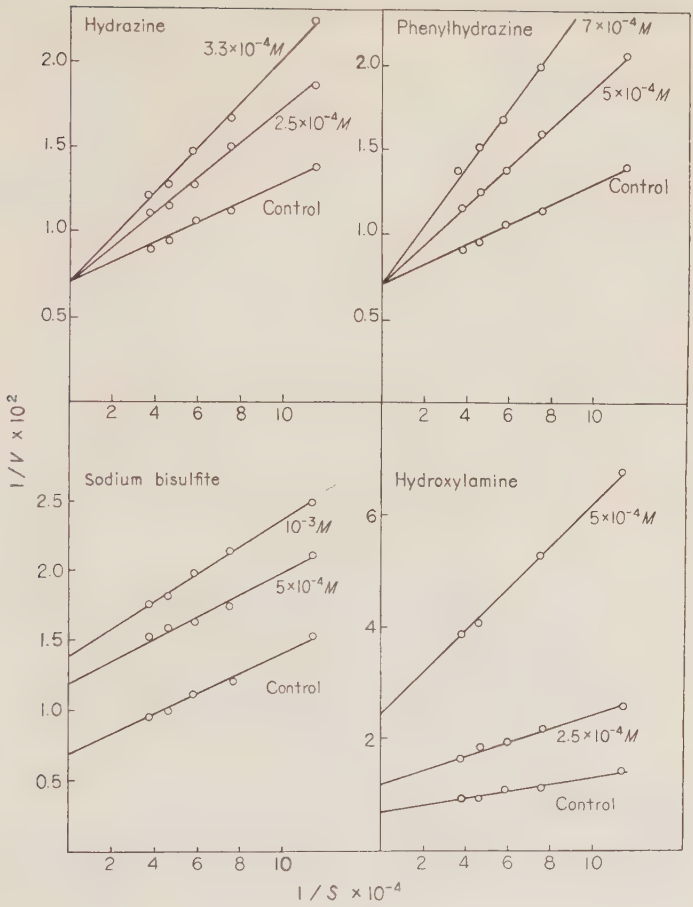

FIG. 1. Nature of inhibition between aldehyde reagents and cytochrome c. Ordinate; Reciprocal of the oxygen uptake in the first 15 minutes; abscissa; Reciprocal of the molar concentration of cytochrome c in the reaction mixture.

Effect of Aldehyde Reagents on the Spectra of Cytochrome a

i) Effect on Cytochrome a—When cytochrome a, in 0.1*M* phosphate buffer containing 1 per cent Emasol 4130 at pH 7.4, was incubated with $10^{-2}M$ hydroxylamine for 30 minutes at room temperature, the spectrum of the oxidized form was not changed. That of the reduced form shifted markedly as shown in Fig. 2; its α-peak shifted from 605 mμ to 603 mμ and its γ-peak from 444 mμ to 433 mμ.

Under the same conditions, there was no marked shift in the position of the peaks on addition of the other reagents though the absorbancy decreased slightly in the region of the γ-band and increased slightly in the region of the α-band. Furthermore, no change in the absorption spectrum

could be observed by preincubation for a long time with the reagents.

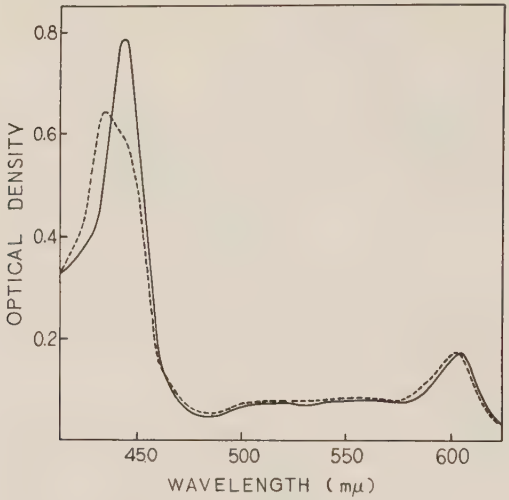

FIG. 2. Effect of hydroxylamine on cytochrome a. Experimental conditions as described in the text. Reduced cytochrome a was obtained by the addition of a trace of sodium dithionite. Solid line, reduced cytochrome a; dotted line, reduced cytochrome a in the presence of hydroxylamine.

ii) *Effect on Hemin a*—To 1.0 ml. of hemin a in pyridine, 1.7 ml. of distilled water and 0.3 ml. of various aldehyde reagents at a concentration of 0.1*M* were added. The mixture was allowed to stand for 30 minutes at room temperature. After addition of sodium dithionite to reduce the hemin a fully, the pH was adjusted to about 10 with 1 *N* NaOH. The hemochromogen of heme a obtained after reaction with aldehyde reagent was then studied. Heme a reacted with hydroxylamine and hydrazine, the absorption band shifting considerably towards shorter wavelengths. This is in partial agreement with the results of Lemberg *et al.* (*4*). With sodium bisulfite-treated hemin a, there was no marked shift in the position of the peaks, but the absorbancy decreased. The reaction of hemin a with phenylhydrazine was difficult to study because the hemin a was rapidly decomposed on incubation under these conditions.

iii) *Effect on Porphyrin a*—Lemberg *et al.* showed the presence of a formyl group in porphyrin a from the effect of aldehyde reagents on its spectral properties. To confirm this, porphyrin a was prepared from highly purified cytochrome a. To a solution of the porphyrin a in pyridine, a few mg. of a mixture of equivalent amounts of solid aldehyde reagent and sodium carbonate was added. These solids were dissolved by gentle rotation, and the solution allowed to stand for 30 minutes at room temperature.

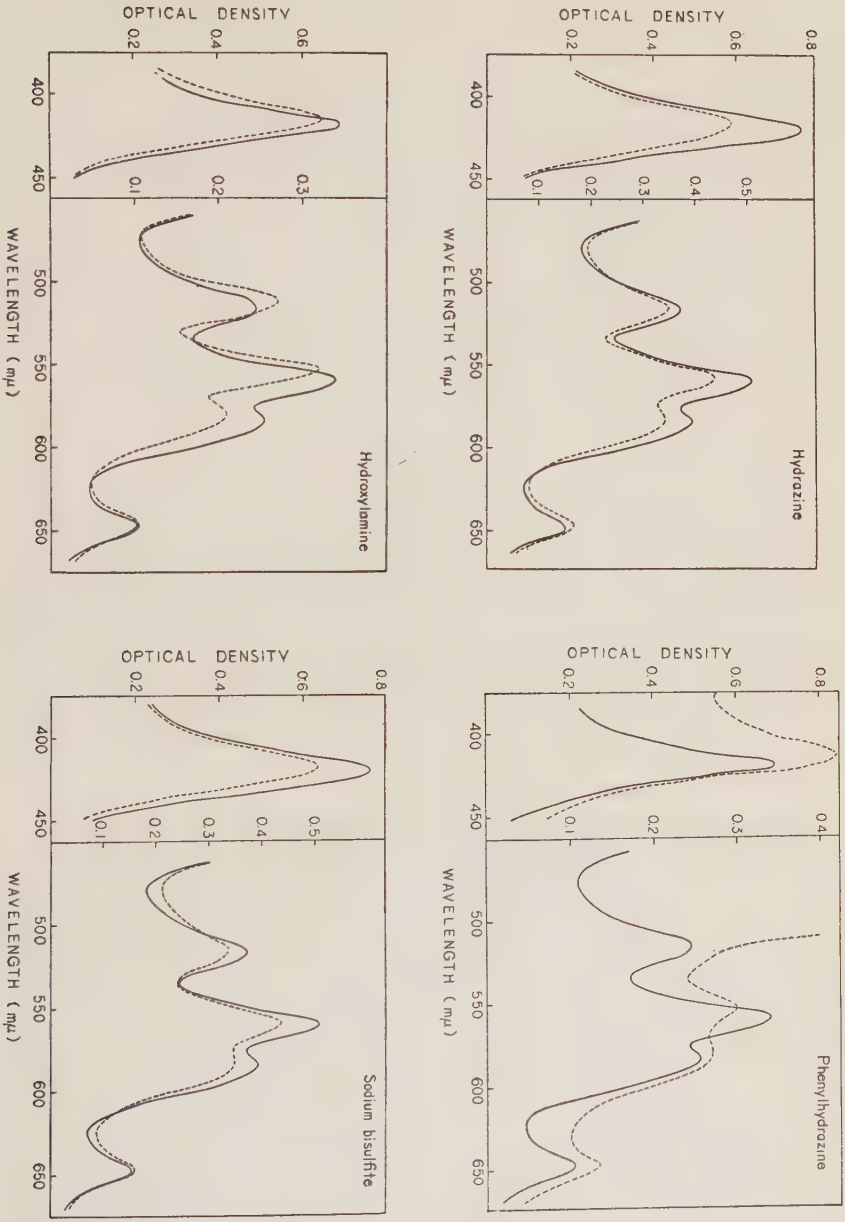

FIG. 3. Effect of aldehyde reagents on porphyrin a.

Experimental conditions as described in the text. Solid line, porphyrin a; dotted line, porphyrin a in the presence of aldehyde reagents.

As shown in Fig. 3, porphyrin a reacted with the aldehyde reagents resulting in a shift in the absorption bands towards shorter wavelengths. This result is in good agreement with those of Lemberg (*4*, *5*) and Oliver and Rawlinson (*3*). The shift of absorption band of porphyrin a with phenylhydrazine was not accurate in shorter wavelengths than 500 mμ because of the color development of phenylhydrazine in pyridine in the above treatment. The spectral change of porphyrin a with sodium bisulfite could only be observed in a dilute pyridine solution because of the insolubility of sodium bisulfite in pyridine.

The spectral changes in the above reaction are probably chiefly due to the formation of an oxime or hydrazone with the formyl group of porphyrin a. The shift of the absorption bands during oxime or hydrazone formation is typical of formyl-substituted porphyrins such as chlorocruoroporphyrin and porphyrin a, but is not given by protoporphyrin.

DISCUSSION

The results presented here indicate that aldehyde reagents such as hydroxylamine, phenylhydrazine, hydrazine, and sodium bisulfite are effective inhibitors of the cytochrome oxidase and that the most likely point of attack of these inhibitors is the formyl group of cytochrome a. However, when cytochrome a reacts with hydroxylamine, the latter may combine with the heme iron. This is suggested from the fact that the inhibition by hydroxylamine is non-competitive with cytochrome c and that the alteration in the absorption spectrum in the presence of hydroxylamine is closely similar to that with carbon monoxide or nitric oxide (*21*). It has been generally believed that hydroxylamine inhibits cytochrome oxidase by formation of a heme iron-hydroxylamine complex. This assumption on the inhibition mechanism was not modified even when the presence of a formyl side chain in porphyrin a was established by Lemberg *et al.* Sevag *et al.* (*22*) reported that hydroxylamine can combine both with heme and non-heme enzyme systems. Since hydroxylamine reacts with both hemin a and porphyrin a and rapidly forms an oxime, it seems likely that both the heme iron and the formyl group must be involved in its attachment. As discussed above, the effect of hydroxylamine is very complicated, while, the mode of inhibition of the other reagents is still obscure. In order to clarify the above problem, catalase was chosen as a heme enzyme without a formyl group, and the effect of these reagents on its activity was examined. The results indicated that aldehyde reagents other than hydroxylamine have no effect. Thus these reagents, other than hydroxylamine, do not seem to react with heme iron. Based on the above results, it seems that the formyl group in cytochrome a is the site of binding to aldehyde reagents. The final solution of these problems must await investigations on the properties of cytochrome a reconstructed by combination of the protein moiety with heme a and modified by these reagents.

Numerous studies on the mode of action of pyridoxal phosphate as a coenzyme for amino acid decarboxylase have implicated that the combination of amino acid with the formyl group of pyridoxal phosphate would occur through the α-amino group probably with the formation of a Schiff's base. As reported in the previous paper of this series (*25*), the cytochrome oxidase reaction involves a complex formation of cytochrome a with cytochrome c. Therefore, it is important to find the site of combination of cytochrome a and c, and it is of interest that the inhibition of cytochrome oxidase by hydrazine or phenylhydrazine is competitive with cytochrome c. It is well known that cytochrome c is a strongly basic protein with an isoelectric point at about 10.5 and lysine constitutes 20 per cent of the total amino acid residues. As indicated by the above findings, the formyl group of cytochrome a may be one point of attachment of cytochrome c in the cytochrome oxidase reaction. This possibility may be partly investigated by studying the catalytic and other properties of cytochrome c chemically modified by substitution on the functional group. Minakami *et al.* (*23*) reported that cytochrome c acetylated to block the amino groups was not active in the succinic oxidase system. We have also found in preliminary experiments that acetylated or succinylated cytochrome c exerts no cytochrome oxidase action in regard to cytochrome a (Okunuki *et al.* unpublished data). Smith and Conrad (*24*) reported that other basic proteins besides cytochrome c, notably salmine, have a greater inhibitory effect on the cytochrome oxidase reaction. These observations strongly support the above supposition. Of course the proposed reaction mechanism is hypothetical and needs further experimental confirmation.

SUMMARY

1. Cytochrome oxidase activity is strongly inhibited by aldehyde reagents such as hydazine, phenylhydrazine, hydroxylamine and sodium bisulfite at a concentration of $10^{-3}M$. The inhibition occurred rapidly and was readily reversed by dialysis.

2. Analysis of the nature of the inhibition suggests that there are both competitive and non-competitive inhibitors with cytochrome c.

3. The absorption spectrum of reduced cytochrome a changes towards shorter wavelengths in the presence of hydroxylamine, while there is no marked shift in the position of the peaks on addition of the other aldehyde reagents.

4. Hemin a and porphyrin a shift their absorption bands towards shorter wavelengths on addition of aldehyde reagents. These shifts in the absorption bands are attributable to the formation of an oxime or a hydrazone with the formyl group.

5. The role of the formyl group of cytochrome a in the cytochrome oxidase reaction is discussed on the basis of these findings.

The authors thank Messrs. Y. Orii and K. Ohnishi for their help and discus-

sion during this investigation.

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STUDIES ON CODEHYDROGENASE

II. AN IMPROVED METHOD FOR THE PREPARATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE FROM BAKER'S YEAST USING ION EXCHANGE RESINS

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Some years ago, a simplified method for the preparation of diphosphopyridine nucleotide (DPN) using a cation exchange resin (Kaken cation exchange resin) was developed in our laboratory (*1*). However, this particular resin is not commercially available.

The present report shows that another cation exchange resin (Duolite C-10) which is readily available commercially is as satisfactory as the Kaken resin in the method. Initial treatment of the yeast extract with an anion exchange resin (Duolite A-2) was found to facilitate the purification. By so doing highly purified DPN could be obtainable more easily with better yield than in the previous method, and without the use of heavy metals.

EXPERIMENTAL

Assay of DPN—DPN was determined with alcohol dehydrogenase (*2*). Its purity was calculated from the ratio of the difference in extinction of the oxidized and reduced forms at 340 mμ, to the extinction at 260 mμ of the oxidized form.

Preparation of Resin Columns—Three kinds of ion exchange resins, Duolite A-2, Duolite C-10 and Duolite C-25, were used. Resin of 60 to 120 mesh was used in each case.

Duolite A-2 (400 ml.) was treated with 1 *N* NaOH (2 liters) at room temperature for 3 to 5 hours. After washing with a large volume of water, the OH-form resin was converted to the acetate form by passing 1 *N* acetic acid (3 liters) through the resin bed which was again washed with water.

The two cation exchange resins, Duolite C-10 and C-25, were treated in the same way. The resins (400 ml. of C-10 and 200 ml. of C-25) were treated with 2 *N* NaOH (2 liters) at 80° to 90° for 1 hour, washed with water and converted to the H-form with 4 *N* HCl (2 liters).

Suspensions of these resins in water were transferred to a glass chromatography tube (inner diameter about 3 cm., height about 70 cm.). The Duolite C-25 was packed to half this height.

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Purification of DPN from Baker's Yeast; *Step 1. Extraction*—Two kg. of fresh Baker's yeast cake* were crumbled and gradually suspended in 1.6 liters of water at 85° to 90° by mechanical stirring. After keeping it at 85° for 10 minutes, 400 g. of coarse celite** were added, and the suspension was rapidly cooled to room temperature in a water bath and filtered through a Buchner funnel, using suction. The residue on the funnel was resuspended in 800 ml. of water and filtered again. The two filtrates were combined.

Step 2. Pretreatment of the Yeast Extract with Duolite A-2—The extract obtained in step 1 above, contained much yellow pigment and impurities. To remove these, it was passed through a Duolite A-2 column at a flow rate of 1 liter per hour. About the first 800 ml. of effluent contained very little DPN (Fig. 1). This fraction was separated from the main effluent which contained most of the DPN. It was adjusted to pH 6.5 with conc. NH_4OH and was used to wash the column after all the extract had been passed through.

Step 3. Adsorption and Elution of DPN on a Duolite C-10 Column—The effluent obtained in step 2 above, was adjusted to pH 2.5 with conc. HCl and then passed through a Duolite C-10 column at a flow rate of 1 liter per hour. The DPN was strongly adsorbed on the resin. After adsorption, the column was first washed with 400 ml. of 0.1 *M* NH_4Cl solution, adjusted to pH 2.5 with conc. HCl, and then with 0.3 *M* NH_4Cl solution of the same pH until the adsorbed DPN began to be eluted. About 500 to 700 ml. of the latter solution was usually required before the DPN began to appear. Ammonium acetate buffer at pH 4.5 containing 0.05 *N* NH_4^+ was passed through the washed column to elute the DPN. The initial effluent which contained little or no DPN (ca. 800 ml.) was discarded and the following eluate (ca. 1200 ml.) containing DPN was collected (Fig. 2). The pH of the effluent usually changed from 4.0 to 4.5 when DPN began to be eluted and this pH change was used as an indicator to separate the above two fractions (Fig. 2).

Step 4. Concentration of DPN by a Duolite C-25 Resin Column—The eluate collected at step 3 was adjusted to pH 2.5 with HCl and passed through a Duolite C-25 column at a flow rate of 1 liter per hour to absorb the DPN. 0.5 *M* CH_3COONH_4 solution was passed through the column in a similar way to step 3 and the part of the effluent containing DPN (ca. 200 ml.) was collected.

Step 5. Alcohol Precipitation—The concentrated DPN solution obtained from the Duolite C-25 column was adjusted to pH 3.0 with 6 *N* HCl. Ten volumes of 99 per cent ethanol was added and the solution was kept at 5° overnight. The white precipitate was collected by centrifugation and washed twice with 95 per cent ethanol, and then successively with absolute ethanol, an ethanol-ethyl ether mixture and ethyl ether. It was then dried *in vacuo*.

RESULTS AND DISCUSSION

In the previous method (*1*) DPN was directly adsorbed on a special cation exchange resin (Kaken resin) from an acidified yeast extract without pretreatment and was then eluted from the column with an alkaline buffer. Since the original yeast extract contains many impurities besides DPN and the above cation exchange resin is not commercially available, this method is not convenient for routine work.

* Fresh Baker's yeast cake was kindly supplied by the Oriental Yeast Co. Ltd.

** Radio-Lite, Rapid Flow, from the Syowa Kagaku Co. Ltd.

In the present method, yellow pigments and some other nucleotides were removed from the yeast extract by passage through a Duolite A-2 column prior to treatment with the cation exchange resin. Although Duolite A-2 can adsorb some DPN under the conditions used, the affinity of the resin for DPN is not so strong as that for many impurities in the yeast extract and almost all of DPN once adsorbed on the resin was displaced by the impurities when a large volume of the extract was passed through the column. Thus, if a suitable volume of the extract was passed through the column, virtual complete recovery of DPN was achieved and many impurities were adsorbed on the column (Fig. 1). By this treatment the purity of the DPN was increased more than 4 fold (Table I).

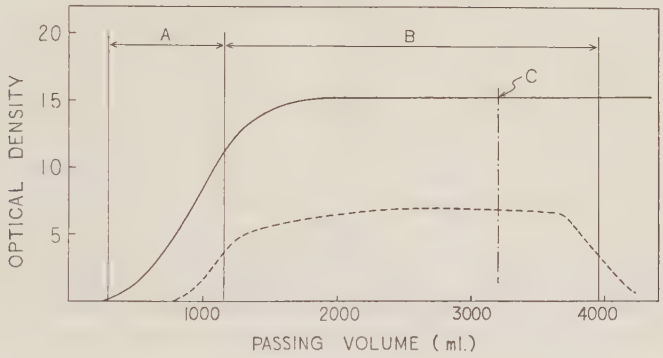

FIG. 1. Pretreatment of the yeast extract by a Duolite A-2 column. Resin column, 3.2×50 cm. Fraction volume, 200 ml. —— extinction at 260 mμ, ----- difference in extinction between oxidized and reduced DPN at $340\,\mathrm{m}\mu \times \frac{16.5}{6.25}$

A: initial effluent, B: main effluent, C: washed with the initial effluent A.

The solution obtained by this treatment contained a little yellow pigment and this impurity was easily removed by the following Duolite C-10 treatment. The DPN adsorbed on the Duolite C-10 column was eluted with ammonium acetate buffer at pH 4.5 leaving this yellow pigment and some other impurities on the column. These could be eluted with alkali. Thus the purity of the DPN was appreciably increased. With the Kaken resin, an alkaline buffer solution is required for elution of DPN and thus some yellow pigment is eluted with the DPN.

Since a large volume of eluate had to be eluted from column to recover the DPN, the latter could be concentrated on the Duolite C-25 column. As mentioned below, Duolite C-25 is a suitable resin for this concentration procedure. Duolite C-10 could also be used, but with this resin DPN was collected at a lower concentration and larger amouuts of resin was required.

It had been reported that of cation exchange resins only polyphenol resins can adsorb much DPN from an acidified yeast extract. However, if

the extract is pretreated with Duolite A-2 as in the present method, considerable DPN is adsorbed on the polystylen polymer, Duolite C-25. In this case, Duolite C-10, a phenolic polymer still showed a higher capacity for DPN than Duolite C-25 (Fig. 3, A.). However, when the eluate from the

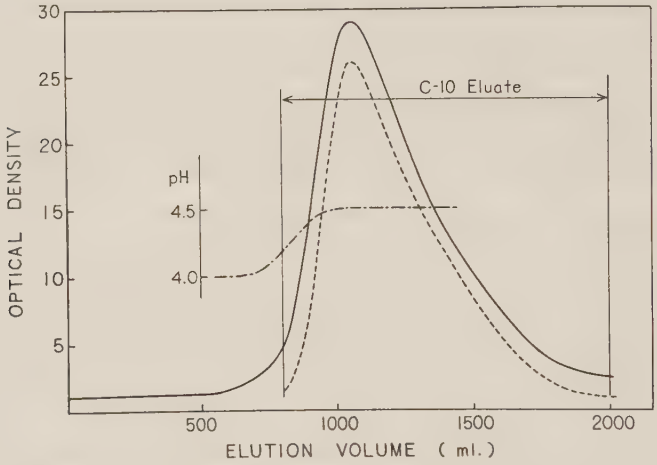

FIG. 2. Elution of DPN from a column of Duolite C-10 with acetate buffer.
Resin column, 3.2×50 cm. (the H-form) Fraction volume, 100 ml. —— extinction at 260 mμ, ----- difference in extinction between oxidized and reduced DPN at 340 mμ $\times \frac{16.5}{6.25}$

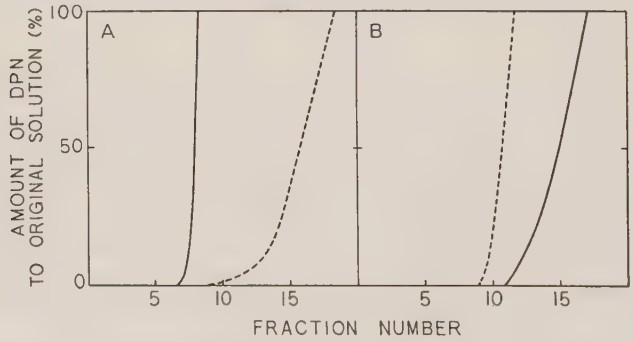

FIG. 3. Comparison of DPN adsorption onto Duolite C-10 and C-25.
(A). Eluate from the A-2 column was passed through each column.
(B). C-10 eluate was passed through.
Resin columns, 0.8×4.0 cm. Fraction volume, 2 ml. ----- Duolite C-10, —— Duolite C-25. DPN was estimated at 340 mμ.

Duolite C-10 column was passed through the above 2 resin columns, Duolite C-25 showed a higher capacity for DPN than Duolite C-10 (Fig. 3, B.). This indicates that the adsorption of DPN onto Duolite C-25 is affected to

a greater extent by impurities than the adsorption onto Duolite C-10. Therefore, Duolite C-10 is suitable to adsorb DPN from the solution containing many impurities, and Duolite C-25 is suitable for use with more purified solutions.

Table I

Summary of the Purification of DPN

	Volume	DPN (μmoles)	Recovery (%)	E_{340}/E_{260}
Baker's yeast	2 kg.			
Extract	2890 ml.	875	100	0.025
A-2 passed soln.	2660	840	94	0.110
C-10 eluate	1175	822	94	0.310
C-25 eluate	200	770	88	0.320
Dried DPN	560 mg.	700	80	0.320
Pure DPN				0.379

Table I shows a summary of a tipical purification of DPN according to the present method. Although dried DPN was about 85 per cent pure in this case, it is not difficult to obtain DPN which is 90 per cent or more pure. A convenient amount of yeast to use for this method is 10 kg.

SUMMARY

Highly purified DPN was prepared in good yield by a simple method, in which the main procedures were carried out by ion exchange resins. About 500 to 600 mg. of DPN was obtained from 2 kg. of fresh Baker's yeast by this method with 85 to 90 per cent purity.

The adsorption of DPN by two types of cation exchange resins, the polyphenol resin, Duolite C-10, and the polystylene resin, Duolite C-25 was compared under different conditions.

The authors wish to express their thanks to Dr. B. Hagihara, Dr. K. Tagawa and Dr. I. Sekuzu for their kind advice and helpful discussion during the present investigations and also to the Oriental Yeast Co. Ltd. for a gift of Baker's yeast.

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INTESTINAL ABSORPTION OF AMINO ACIDS

IV. PARTICIPATION OF PYRIDOXAL PHOSPHATE IN THE ACTIVE TRANSFER OF L-AMINO ACIDS THROUGH THE INTESTINAL WALL

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(Received for publication, June 28, 1960)

In the previous report (*1*), using the *in vivo* method, pyridoxine was shown to stimulate intestinal absorption of L-amino acids in rats made V. B_6 deficient with L-penicillamine. Pyridoxine did not accelerate the rate of absorption of D-isomers, which passed through by simple diffusion.

Jacobs and Hillman (*2*) showed by the *in situ* technique, that pyridoxal phosphate partially counteracted the inhibitory effect of 2, 4-dinitrophenol (DNP) on L-methionine absorption, whereas pyridoxine did not. In this paper using the everted intestine *in vitro*, it is shown that only pyridoxal phosphate counteracts the inhibition of active transfer of L-amino acids caused by DNP. Furthermore, from comparative studies on the competitive and non-competitive mechanisms of the effects of L- and D-penicillamine on pyridoxal phosphate requiring enzymes and on the intestinal absorption of L-amino acids, we propose that there is some carrier system associated with pyridoxal phosphate during the active transfer of L-amino acids through the intestinal wall.

EXPERIMENTAL

Young male albino rats weighing 120–150 g. were used. They are fasted for about 30 hours and then anaesthetized with ether before the experiments. For *in vitro* experiments, about 10 cm. lengths of everted intestine were incubated in Krebs-Ringer bicarbonate buffer containing 0.2 per cent glucose. The reaction tube was as described previously (*1*). The experimental conditions are shown in Fig. 1. *In vivo* studies were carried out by the perfusion technique. The small intestine was exposed by laparatomy, and the section of the gut from the jejunum to the ileocaecal region was divided into two equal lengths which were placed in the circulation apparatus, as reported previously (*1*). Some animals received intraperitoneal injection of 10 mg. of L- or D-penicillamine per 100 g. body weight at a fixed time prior to the perfusion (Fig. 2). Histidine was measured by the method of Macpherson (*3*) and methionine by the improved method of McCarthy and Sullivan as reported by Bolling (*4*).

Tryptophanase was partially purified from an acetone powder of *E. Coli.* adapted to tryptophan. The purification methods and the experimental conditions are shown in

Table I and II. The enzyme activity was determined, using a slight modification of Goto's method (*5*), by the amount of indole formed. Thus, after treating the reaction mixture with trichloracetic acid at a final concentration of 5 per cent, 10 ml. of petroleum ether were added to the solution and then the mixture was shaken in a separating funnel for two minutes to extract the indole. The petroleum ether layer was taken and 5 ml. of aldehyde reagent was added and the mixture was incubated for one hour at 37°. After cooling the tube in an ice water, the colour which had developed in the acid ethanol layer was measured at 560 mμ in a Beckmann-type spectrophotometer. The aldehyde reagent used for the estimation of indole was composed of 30 ml. of concentrated sulphuric acid, 200 ml. of ethanol and 2 g. of 2,4-dimethyl-*p*-aminobenzaldehyde.

Transaminase in a rat liver homogenate was measured by Caldwell and Mac Henry's procedure (*6*). The experimental conditions was shown in Fig. 6.

Protein was determined by the method of Robinson *et al.* (*7*). Penicillamine HCl was obtained from California Foundation. Co. Ltd., (U.S.A.) and pyridoxal phosphate from Wakamoto Co. Ltd., (Japan).

RESULTS AND DISCUSSION

Inhibition of Transfer of L-*Histidine by DNP in vitro and Reversal of Inhibition by Pyridoxal Phosphate*—The rate of transfer of L-histidine from the mucosa

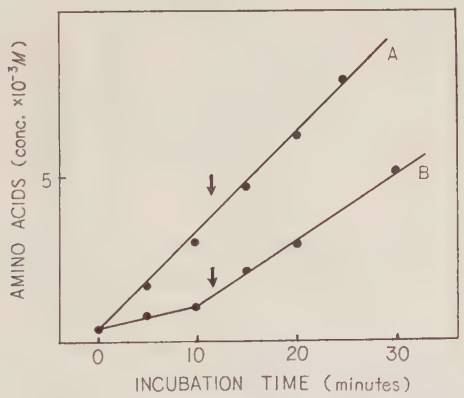

FIG. 1. Inhibitory effect of DNP on the transfer of L-histidine and reversal of inhibition by pyridoxal phosphate.

About 10 cm. lengths of everted intestine were incubated at 37° in a Wilson apparatus. Krebs-Ringer bicarbonate buffer containing 0.2 per cent glucose (pH 7.4) was present on both sides of the sac and 5 m*M*. of L-histidine was added to the external fluid. The reaction tube was preincubated for 10 minutes and then 0.05 ml. samples were taken out at intervals for assay. One mg. of pyridoxal phosphate was added at the time indicated by the arrows. Curve A, indicates the normal rate of transfer of L-histidine from the mucosa to the serosa. In curve B, DNP was added to the fluid outside the sac at a final concentration of 0.5×10^{-4} *M*.

to the serosa was measured by Wilson's method as described below. At the start of the experiment, Krebs-Ringer bicarbonate solution (PH 7.4)

containing 0.2 per cent glucose was on both sides of the intestine, and 5 m*M*. of L-histidine was added to the mucosal fluid. After ten minutes preincubation at 37°, 0.05 ml. aliquots of the serosal fluid were taken out at five minutes intervals.

As shown in Fig. 1, curve A, concentration of L-histidine in the serosal fluid steadily increased for 30 minutes from 0.5 m*M*. to 10 m*M*. When 0.5×10^{-4} *M*. of DNP was added to the mucosal fluid, the rate of L-histidine transfer was decreased to one third of that of the control. The addition of one mg. of pyridoxal phosphate, 12 minutes after the start of the experiment, reversed the DNP inhibition significantly as seen in Fig. 1, curve B.

One mg. of pyridoxal, pyridoxine HCl or thiamine pyrophosphate had no effect on the inhibition caused by DNP. Furthermore, when D-histidine was used in place of L-histidine, its slow transfer was not influenced by DNP or pyridoxal phosphate, since absorption of the D-isomer was due only to free diffusion, as reported previously (*1*).

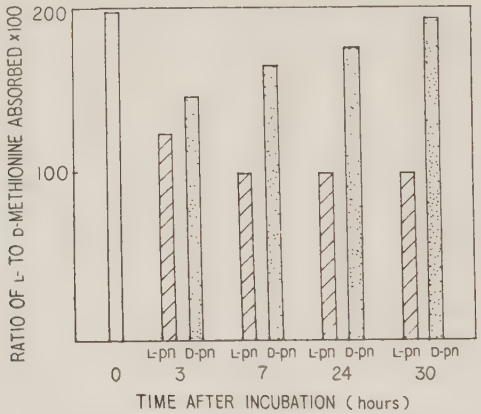

FIG. 2. Inhibitory effect of L- and D-penicillamine on intestinal absorption of L-methionine *in vivo*.

The *in vivo* perfusion technique was used. The circulation fluid was Krebs-Ringer bicarbonate solution (pH 7.4) containing 0.2 per cent glucose and D- or L-methionine at a concentration of 10 m*M*. The disappearance of the amino acids in the lumen was measured after 45 minutes. The units on the abscissa indicate the time after intraperitoneal injection of 10 mg. of L- or D-penicillamine in Krebs-Ringer solution per 100 g. body weight. The ordinate indicates the ratio of L- to D-methionine absorbed, taking 100 as the value of the D-isomer. Therefore, the columns indicate the absorption of L-methionine in relation to that of D-methionine. L-pn indicates L-methionine absorption after injection of L-penicillamine. D-pn indicates L-methionine absorption after injection of D-penicillamine.

Of special interest in this connection is the fact (*8*) that the rate of

consumption of ATP by the erythrocyte membrane is accelerated during the active transfer of sodium and potassium ions. From the above results, it seems likely that ATP participates in the active transfer by causing regeneration of pyridoxal phosphate. Moreover, pyridoxal kinase was detectable in intestinal tissue. Our studies on the breakdown of pyridoxal phosphate during L-amino acid absorption will be published shortly.

The Inhibitory Effect of D- *and* L-*Penicillamine on* L-*Methionine Absorption in vivo*—In 1960, Suda *et al.* (*1*) reported that *in vivo*, the rate of L-amino acid absorption was as low as that of the D-isomer in rats made $V.B_6$ deficient with L-penicillamine, and administration of $V.B_6$ restored the rate to normal. In the experiment presented here, the effect of L- and D-penicillamine on absorption were compared.

Ten mg. of D-penicillamine per 100 g. body weight was injected intraperitoneally into albino rats. At defined times—3, 7, 24 and 30 hours—after the injection, two sections of the small intestine were placed in the circulation apparatus. To reduce biological variations in the *in vivo* studies, one of the two sections of the gut was used to study D-methionine absorption and the another to study L-methionine absorption.

As may be seen in Fig. 2, the rate of L-methionine absorption gradually diminished to that of the D-isomer during about 7 hours, following the administration of L-penicillamine. It remained at this level for more than 30 hours. In contrast to this, D-penicillamine caused a slight decrease in the rate, shortly after injection, but the normal rate was soon regained.

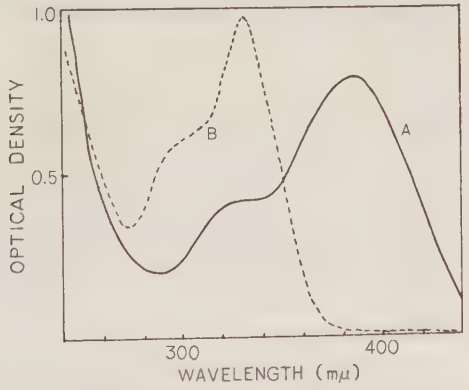

FIG. 3. Absorption spectrum of pyridoxal phosphate and the thiazolidine compound formed with penicillamine.

A: Absorption spectrum of pyridoxal phosphate (0.2 μmoles/ml.) in 0.1 *M* phosphate buffer (pH 7.4).

B: Absorption spectrum of the thiazolidine compound formed when pyridoxal phosphate (0.2 μmoles/ml.) was incubated with D- or L-penicillamine (0.3 μmoles/ml.) in 0.1 *M* phosphate buffer (pH 7.4) at 37° for one hour.

Relation of Pyridoxal Phosphate and Penicillamine—It was reported by du

Vigneaud *et al.* (*9*, *10*, *11*) that pyridoxal phosphate formed a thiazolidine compound with L-penicillamine non-enzymatically. This compound was also formed with D-penicillamine as shown in Fig. 3.

This does not seem compatible with the observation that only L-penicillamine inhibited the active transfer of L-amino acids, since both isomers of penicillamine can react with pyridoxal phosphate.

Therefore, an attempt was made to elucidate this phenomenon with an enzyme requiring pyridoxal phosphate as a cofactor. An enzyme which could be easily separated from its cofactor was required and so tryptophanase was chosen. Its purification and the experimental conditions are summarized in Table I and II.

TABLE I

Purification of Tryptophanase

E. Coli. K_{12} was grown in ordinary peptone-bouillon medium for 24 hours at 30°. The harvested cells were adapted by shaking for 7 hours at 30° in 10 liters of a medium containing 40 g. of DL-tryptophan and 20 g. each of casamino acid, yeast extract, NaCl, K_2HPO_4 and glucose. An acetone powder was prepared from the adapted cells. Two g. of the acetone powder were suspended in 50 ml. of distilled water, and the mixture was stored overnight at 4°, and then centrifuged at 30,000 r.p.m. for 20 minutes. The supernatant thus obtained is designated as (1) below.

Supernatant (1)

↓ Streptomycine added to a concentration of 0.5 per cent. Mixture adjusted to pH 5.0 with 10^{-1} *M* acetic acid, and centrifuged. Supernatant re-adjusted to pH 7.4 with 10 per cent ammonia.

Supernatant (2)

↓ Incubated for 5 minutes at 50°, centrifuged.

Supernatant (3)

↓ Adjusted to pH 7.0 with 10^{-1} *M* acetic acid. Ca-PO_4 gel added in the gel/protein ratio of 0.5. Mixture centrifuged. Supernatant readjusted to pH 7.4 with 10 per cent ammonia.

Supernatant (4)

↓ Adjusted to pH 6.0 with 10^{-1} *M* acetic acid. Saturated $(NH_4)_2SO_4$ added to 40 per cent saturation. Mixture stored overnight in an ice chest. Centrifuged. Solution fractionated between 55–65 per cent saturated $(NH_4)_2SO_4$. Precipitate dissolved in 0.1 *M* phosphate buffer (pH 7.4) and dialyzed against 2 liters of distilled water containing 0.5 per cent mercaptoethanol overnight. Centrifuged.

Supernatant (5) : Purified enzyme.

Mercaptoethanol was added to the solutions in each step at a final concentration of 0.5 per cent. All the above mentioned procedures were carried out at 4°.

TABLE II

Specific Activity and Yield of Tryptophanase at Each Step of Purification

Supernatant	Total activity, as units of enzyme	Total protein	Specific activity	Yield
	unit	*mg.*	units per mg. protein	%
(1)	3890	460	8.4	100
(2)	3690	345	10.7	95
(3)	3230	258	12.5	83
(4)	2770	160	17.3	71
(5)	1750	20	87.5	45

The reaction mixture contained 5 μmoles of L-tryptophan, 20 μmoles of $(NH_4)_2SO_4$, 20 μmoles of mercaptoethanol, 2 μg. of pyridoxal phosphate, 80 μmoles of phosphate buffer (pH 8.0) and the purified enzyme (about 100 μg. protein). The total volume was adjusted to 4 ml. with distilled water. The tubes were incubated at 37° for two minutes, and then the amount of indole was measured as described in the text. One unit of enzyme was that which formed 10^{-2} μmoles of indole per minute under the above conditions.

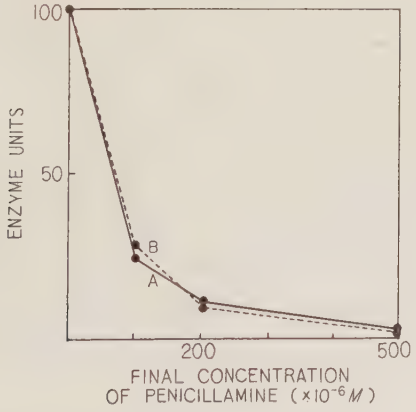

FIG. 4. Inhibition of tryptophanase by L- and D-penicillamine after formation of thiazolidine compounds with the enzyme.

Experimental conditions are as in Table II. Full activity was taken as 100 per cent. Ten minutes after preincubation of 2 μg. of pyridoxal phosphate with various concentrations of penicillamine at 37°, the apoenzyme of tryptophanase was added. The mixture was incubated for another 10 minutes at the same temperature. Then, the substrate (5 μmoles) was added. Two μg. of pyridoxal phosphate was sufficient to half saturate the apoenzyme. Curve A; With L-penicillamine. Curve B; With D-penicillamine.

The enzyme solution at the final stage of purification in Table II was coenzyme free, and about 0.1 mg. of this preparation gave measurable activity after 2 minutes incubation.

As shown in Fig. 4, 2 μg. of pyridoxal phosphate was preincubated for 10 minutes at 37° with each isomer of penicillamine to form the respective compounds, and then excess apoenzyme was added, (2 μg. of the coenzyme was shown to half saturate the enzyme), The reaction mixture was incubated for another 10 minutes at the same temperature, and then 5 μmoles of L-tryptophan was added. After two minutes, the indole formed was measured with aldehyde reagent as described above. Under these conditions, the inhibitory effect of both isomers of penicillamine was almost the same because a similar complex with pyridoxal phosphate was formed (Fig. 3).

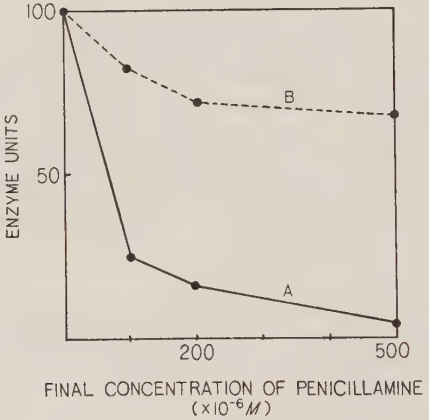

FIG. 5. Inhibition of tryptophanase by L- and D-penicillamine. Conditions as in Table II. Full activity was taken as 100 per cent. Ten minutes after preincubating of tryptophanase with 2 μg. of pyridoxal phosphate at 37°, varying concentrations of penicillamine were added, and mixture was incubated for another ten minutes at the same temperature. Then the substrate was added. Two μg. of pyridoxal phosphate was sufficient to half saturate the apoenzyme.

Curve A; With L-penicillamine. Curve B; With D-penicillamine.

However, the results shown in Fig. 5 are in clear contrast to those in Fig. 4, in which only L-penicillamine counteracted the enzyme activity after the apoenzyme had been incubated with pyridoxal phosphate.

Similar experiments were carried out with transaminase of a rat liver homogenate. In this case, the alanine-glutamic system was used, and the results obtained were compatible with those for tryptophanase except that D-penicillamine showed some activation to the enzyme system, as seen in

Fig. 6. These experiments show that inhibition caused by L-penicillamine

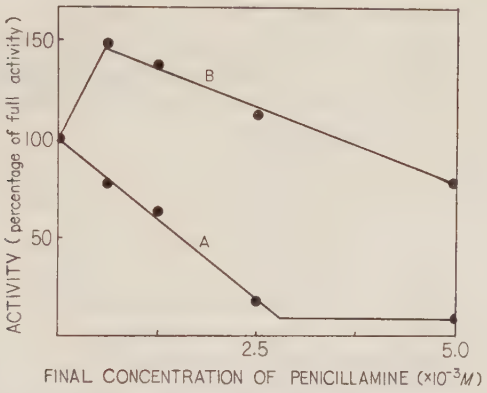

FIG. 6. Inhibition of alanine-glutamic transminase in a rat liver homogenate by penicillamine.

The reaction mixture contained 50 μmoles of L-alanine, 10 μmoles of α-keto-glutarate, various concentrations of penicillamine, 200 μmoles of phosphate buffer (pH 7.4) and a freshly prepared homogenate equivalent to 0.3 mg. of wet liver. The total volume was made up to 2 ml. and the tube was incubated for one hour at 37°. After addition of trichloracetic acid, the pyruvate concentration was measured by Friedemann and Haugen's method (*12*).

Curve A; With L-penicillamine. Curve B; With D-penicillamine.

is not due only to thiazolidine formation with pyridoxal phosphate, but to a modification of the enzyme protein because of its combination with the L-isomer of penicillamine.

Moreover, from comparative studies on the mode of action of penicillamine on the active transfer of L-amino acids and on pyridoxal phosphate requiring enzymes, it seems that, during the active transportation, as with enzymatic reactions, there may be some carrier protein associated with pyridoxal phosphate which is inhibited by L-penicillamine. Since DNP inhibition of the active transfer could only be restored by pyridoxal phosphate, it seems likely that this coenzyme could be regenarated through an energy yielding reaction.

SUMMARY

1. Using everted rat intestines *in vitro*, it was found that the rate of transfer of L-histidine from the mucosa to the serosa was suppressed by DNP and the original rate was restored only by pyridoxal phosphate.

2. Using the perfusion technique *in vivo*, evidence was found that L- and not D-penicillamine inhibited L-methionine absorption.

3. A study was made of the effect of L- and D-penicillamine on

pyridoxal phosphate-requiring enzymes such as tryptophanase and transaminase. Only the L-isomer inhibited enzyme activity strongly, though both isomers could combine with pyridoxal phosphate. After thiazolidine formation, both L- and D-penicillamine strongly inhibited these enzymes.

4. From these results, the hypothesis is proposed that there may be a carrier protein involved in amino acid absorption from the intestine which is associated with pyridoxal phosphate.

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A PROTEOLYTIC ENZYME OF STREPTOMYCES GRISEUS

VI. HYDROLYSIS OF PROTEIN BY STREPOMYCES GRISEUS PROTEASE*

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It has been found that almost all kinds of proteinase have a fairly strict substrate specificity and hydrolyze only those peptide-bonds in the protein which satisfy the respective requirements of each proteinase (*1*). Accordingly, so far as is known, the extent of hydrolysis of protein by any proteinase is limited to 10–30 per cent, and then almost all the resulting digestion products are poly- and oligopeptides.

On the contrary, the present authors found that *Streptomyces griseus* protease (*2*) had a very broad substrate specificity and was capable of hydrolysing various kinds of peptide-bonds in protein until the majority of amino acids constructing the protein are liberated as individual free amino acids. As a result, the extent of hydrolysis of proteins by this protease was estimated to reach upward of 60–90 per cent. Such a strong proteolytic activity inherent in this protease has been never found in any previously isolated proteinase, and is regarded as the most dinstinctive trait of this protease. Thus, the protease is promising to be used for many industrial and scientific applications.

In this paper are presented some experimental results on the proteolytic activity of *Streptomyces griseus* protease.

MATERIALS AND METHODS

Enzymes—Streptomyces griseus protease used for the present work is a highly purified, lyophilized preparation which has been prepared as described before (*3*), and confirmed to be homogeneous by ultracentrifugal, electrophoretic and enzymological analyses (*3*). The pepsin, trypsin, chymotrypsin and *Bacillus subtilis* proteinase (*4*) used were crystalline preparation commercially available, unless otherwise specified.

The unit of proteinase activity was determined by the casein-Folin color method (*2*) and represented by *PU* value (*2*).

Assay for Extent of Hydrolysis of Protein—Using casein and other proteins as a substrate, the extent of hydrolysis of protein was measured by the van Slyke volumetric method (*5*) or by the formol titration method (*3*). In the later case, 5 ml. of 35 per cent formal

* A large part of the data was published in Japanese in the *Reports of the Institute of Physical and Chemical Research*, **35**, 90 (1959).

solution (pH 8.0) was added to 5 ml. of protein hydrolysate (initial protein concentration: 5 per cent) and the mixture was titrated electrometrically with 0.02 *N* sodium hydroxide (end point: pH 8.5).

Method for Hydrolysing Protein—Optimum conditions for completing the hydrolysis of proteins within 24–48 hours by *Streptomyces griseus* protease were decided as follows: 2.5–5.0 *PU* of protease were added per 100 g. of protein (Fig. 1), with a reaction pH of 7–8 (*6*), and a reaction temperature of 35°–60° (Fig. 2).

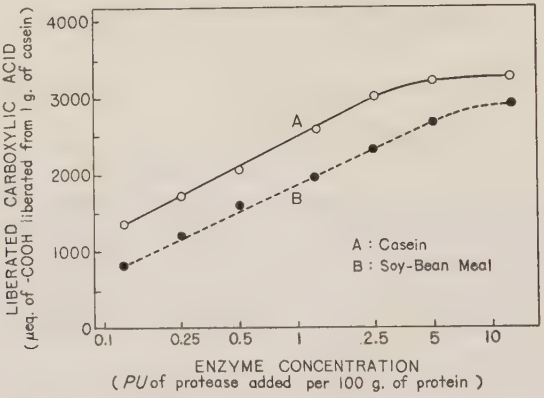

Fig. 1. Relation between enzyme concentration and extent of hydrolysis.

As a substrate, 5% casein solution (curve A) or 5% soy-bean meal suspension (curve B) was used. Reactions were carried out at pH 7.0, at 30° for 24 hours, using a gradient concentration of *Streptomyces griseus* protease.

Extent of hydrolysis was determined by the formol titration method after 24 hours reaction.

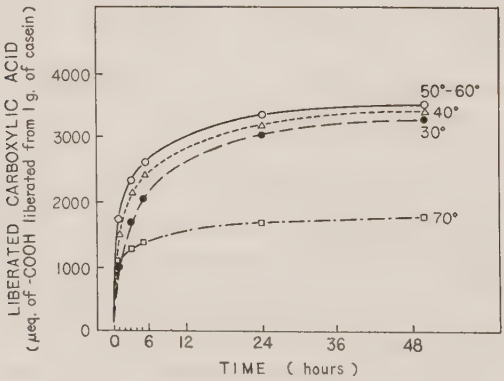

Fig. 2. Relation between reaction temperature and extent of hydrolysis.

Reactions were carried out at various temperatures at pH 7.4 for 48 hours, using 5% casein solution as a substrate. In each case, 2.5 *PU* of *Streptomyces griseus* protease was added per 100 g. of casein.

Extent of hydrolysis was determined by the formol titration method.

When 3–5 per cent of ethanol is previously added to the reaction mixture, contamination with bacteria can be prevented and so the whole reaction can be conveniently accomplished in an open vessel (Table I).

TABLE I

Antiseptic Effect of Ethanol during Hydrolysis of Protein

Conc. of ethanol (%)	Growth of bacteria[1] (turbidity)	Residual proteinase activity[2] (%)
0	##	100
2.5	±	101
5.0	–	98
10.0	–	102

1) After inoculation of a small amount of bacterial culture into 5% casein solution (pH 7.4) or 5% soy-bean meal suspension containing a suitable amount of ethanol, the mixture was kept at 30° for 48 hours. The sign (##) shows a furious growth of bacteria as determined by observation of turbidity, (±) a questionable amount of growth and (–) no growth.

2) After keeping *Streptomyces griseus* protease solution containing a suitable amount of ethanol at 20° for 24 hours, the residual proteinase activity was determined by the casein-Folin color method.

Course of Hydrolysis of Casein by Alternate Use of Various Proteinases—Each 100 ml. aliquot of 5 per cent casein solution containing 0.03 *M* sodium phosphate buffer at pH 7.4 and 5 per cent of ethanol was mixed with one of the following proteinases, *i. e.* 25 mg. of *Streptomyces griseus* protease (correspond to 0.25 *PU* of proteinase activity), 45 mg. of trypsin, 45 mg. of chymotrypsin, 45 mg. of pepsin* or 45 mg. of *Bac. subtilis* proteinase, and allowed to react at 35°. At suitable intervals during the reaction period, 5 ml. of the reaction mixture was transferred into 5 ml. of 35 per cent formol solution and electrometrically titrated with 0.02 *N* sodium hydroxide as previously described. When the titration curve reached a plateau, a corresponding amount of another proteinase or the same proteinase was supplemented to the reaction mixture and the course of hydrolysis was successively followed by the formol titration method under the same conditions as described.

RESULTS AND DISCUSSION

Substrate Specificity of Streptomyces griseus Protease—It was pointed out earlier by Northrop *et al.* (*7*) that an addition of trypsin to casein previously hydrolyzed with chymotrypsin, or of chymotrypsin to casein previously hydrolyzed with trypsin, caused a marked increase in hydrolysis, because the hydrolysis of peptide-bonds of casein by the two proteinases occurs at different linkages. Since the above method seems to be available for preliminary estimation of substrate specificity of a given proteinase, the present authors also attempted to identify the substrate specificity of *Strepto-*

* In case of peptic digestion, the reaction was carried out at pH 2.1.

myces griseus protease with those of trypsin, chymotrypsin, pepsin and *Bac. subtilis* proteinase (*8*, *9*) by drawing a comparison between the hydrolysing courses of casein by alternate use of the above mentioned proteinases.

From the results obtained, it was confirmed that *Streptomyces griseus* protease possessed an extremely broad substrate specificity and so was capable of hpdrolysing casein far more completely than any other proteinase tested. This is shown by the fact that the addition of *Streptomyces griseus* protease to casein previously hydrolyzed with other proteinases causes a remarkable increase in hydrolysis (Fig. 3), whereas there is no indication of increase in hydrolysis by addition of the other proteinases to casein previously hydrolyzed with *Streptomyces griseus* protease (Fig. 4).

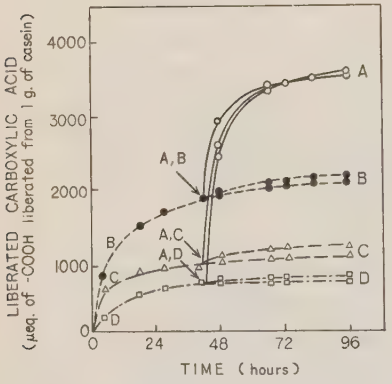

Fig. 3. Hydrolysis of casein by trypsin, chymotrypsin, or *Bac. subtilis* proteinase followed by *Streptomyces griseus* protease.

Using 5% casein solution as a substrate, reactions were carried out at 35°, at pH 7.4. Course of hydrolsis was followed by the formol titration method.

(A) —○— *Streptomyces griseus* protease
(B) ---●--- *Bac. subtilis* proteinase
(C) —△— Trypsin
(D) ·-□-· Chymotrypsin

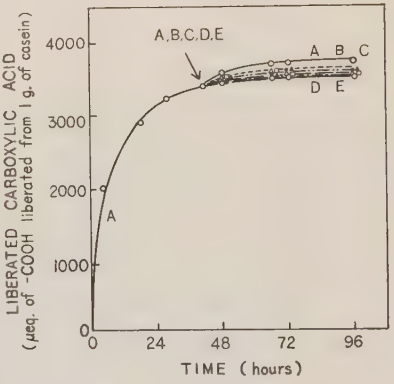

Fig. 4. Hydrolysis of casein by *Streptomyces griseus* protease followed by trypsin, chymotrypsin, pepsin, or *Bac. subtilis* proteinase.

Using 5% casein solution as a substrate, reactions were carried out at 35°, at pH 7.4 (excepting pH 2.1 for pepsin). Course of hydrolysis was followed by the formol titration method.

(A) —○— *Streptomyces griseus* protease
(B) ---●--- *Bac. subtilis* proteinase
(C) —△— Trypsin
(D) ·-□-· Chymotrypsin
(E) ··-▲-·· Pepsin

This information seems to induce a possible assumption that the majority of substrate specificities of trypsin, chymotrypsin, pepsin and *Bac. subtilis* proteinase may be included in that of *Streptomyces griseus* protease. Further detailed experiments, however, are needed in order to confirm the above consideration and this approach is currently being carried out by the authors using many synthetic substrates, such as dipeptides, tripeptides, amino acid-amides, amino acid esters, *etc.*

Extent of Hydrolysis of Proteins by Streptomyces griseus Protease—Owing to its broad substrate specificity described above, *Streptomyces griseus* protease was expected to hydrolyze a large percentage of peptide-bonds in protein.

Using casein, ovalbumin or wheat gluten as a substrate, the extent of hydrolysis of proteins in 72 hours reaction with *Streptomyces griseus* protease was determined by the van Slyke method, and the percentage hydrolysis was calculated and compared with the values of acid hydrolysis of the same proteins set at 100 per cent. The results obtained shows that peptide-bonds were hydrolyzed to the extent of 75 per cent in casein, 87 per cent in ovalbumin and 80 per cent in wheat gluten by *Streptomyces griseus* protease, respectively (Table II).

Table II

Extent of Hydrolysis of Proteins by Streptomyces griseus Protease

Protein	Liberated Amino-Nitrogen[1] (mg. of NH_2-N liberated from 1 g. of protein)		Percentage of Hydrolysis (A/B)×100
	(A) Enzyme Hydrolysis[2]	(B) Acid Hydrolysis[3]	
Casein	69.6 mg.	93.0 mg.	74.8%
Ovalbumin	78.6	89.4	87.0
Wheat Gluten	62.5	78.4	80.0

1) Amino-nitrogen determination was by the method of van Slyke (*5*) in all cases.

2) Five mg. of enzyme containing 0.05 *PU* of proteinase activity was mixed with 20 ml. of 5% protein solution and allowed to react for 72 hours at pH 7.4, at 40°.

3) One volume of 5% protein solution was mixed with 7 volumes of 6*N* HCl and hydrolyzed at 110° for 24 hours.

Table III

Comparison of Extent of Hydrolysis of Casein with Various Proteinases[1]

Proteinase	Amount of Enzyme used	Liberated Carboxylic Acid[2] (μeq. of -COOH liberated from 1 g. of casein)	Relative Ratio	Percentage of Hydrolysis
Streptomyces griseus protease	7 mg.	3,600	1.00	75 %
Trypsin	7	1,120	0.31	23
Chymotrypsin	7	760	0.21	16
Pepsin (crude)	14	1,510	0.42	31
Papain (crude)	70	1,360	0.38	28
,, (crude)	140	1,440	0.40	30
Taka-Diastase	70	1,380	0.38	28
Bac. subtilis proteinase	7	1,960	0.54	40

1) In each case, a described amount of proteinase was mixed with 20 ml. of 5% casein solution, respectively, and allowed to react for 72 hours at 40°, at pH 7.4 (excepting pH 2.1 for pepsin).

2) Carboxylic acid was determined by the formol titration method.

For reference, the magnitudes of extent of hydrolysis of casein with various well-known proteinases in 72 hours reaction were compared with that of *Streptomyces griseus* protease (Table III). In this case, the extent of hydrolysis was measured by the formol titration method and the percentage hydrolysis was computed from the values presented in Table II.

From these data, it has been definity confirmed that the proteolytic activity of *Streptomyces griseus* protease is far stronger than that of any other proteinases, so far as is known in literature. This characteristic seems to be the most distinguishing feature of *Streptomyces griseus* protease and is expected to develop many new industrial and scientific applications of this enzyme.

Amino Acids Liberated from Protein by Streptomyces griseus Protease—As a natural consequence of the fact that *Streptomyces griseus* protease has a very broad substrate specificity and is able to cleave almost all peptide-bonds in protein indiscriminately, the majority of amino acids constructing the protein should be liberated by this enzyme as free amino acids.

In fact, various free amino acids, *e.g.*, glutamic acid, glutamine, aspartic acid, tyrosine, phenylanine, tryptophan, histidine, lysine, arginine, glycine, alanine, valine, leucine (isoleucine), serine, threonine, cysteine, methionine, *etc.*, were detected in the hydrolysate of casein prepared by 72 hours hydrolysis with *Streptomyces griseus* protease, by means of paper chromatography. During the above hydrolysis, a large quantity of tyrosine was observed to precipitate as small needle crystals because of its insolubility.

Quantitative analysis of amino acids liberated from casein or soy-bean glycinin by enzyme hydrolysis was then carried out by means of bio-assay (*10*) and percentage yield of respective amino acids was also computed on the basis of acid hydrolysis (Table IV).

As shown in Table IV, almost all of the amino acids originally constructing the proteins were found to be liberated as free amino acids into the resulting hydrolysates, in good yields. Especially, some kinds of amino acid such as tryptophan, methionine, threonine, cysteine, tyrosine, *etc.*, which are easily decomposed during acid hydrolysis, were recovered more completely in the case of enzyme hydrolysis.

Considering the above data together with the magnitude of the extent of hydrolysis of protein, it is supposed that *Streptomyces griseus* protease may be able to hydrolyze not only proteins but also oligopeptides including di- and tri-peptides. Bergmann *et al.* (*12*) pointed out earlier that proteolytic enzymes should be classified into two groups, *i.e.*, endo-peptidase and exo-peptidase, on the basis of their mode of action. The present protease, however, seems to be a proteolytic enzyme of a new type, which belongs to neither of the above two types. More accurate discussion on this subject will be presented in the next paper, using synthetic oligopeptides as a substrate.

Some Applications of Streptomyces griseus Protease—The above mentioned, excellent proteolytic activity of *Streptomyces griseus* protease is expected to

Table IV

Amino Acids Liberated from Proteins

1. From 100 g. of Casein

Amino acid	(A) Enzyme Hydrolysis[1)]	(B) Acid Hydrolysis[2)]	Yield (A/B)×100
Alanine	1.47 g	2.99 g	49.2 %
Arginine	3.77	5.26	71.6
Aspartic acid	2.78	6.07	45.7
Cysteine	0.49	0.15	—
Glutamic acid	—	10.64	—
Glycine	1.10	1.82	60.5
Histidine	2.40	3.03	79.2
Isoleucine	3.04	5.27	57.7
Leucine	5.98	9.79	61.1
Lysine	5.15	6.99	73.7
Methionine	2.27	2.42	93.8
Phenylalanine	4.19	5.70	73.5
Proline	6.11	9.87	61.9
Serine	3.70	5.27[a)]	70.2
Threonine	2.82	3.58[a)]	78.8
Tryptophan	1.00	0.00	—
Tyrosine	4.46	5.50[a)]	81.1
Valine	4.10	6.73	60.9

2. From 100 g. of Soy-Bean Glycinin

Amino acid	(A) Enzyme Hydrolysis[1)]	(B) Acid Hydrolysis[2)]	Yield (A/B)×100
Alanine	2.17 g.	3.28 g.	62.2 %
Arginine	6.28	7.99	78.6
Aspartic acid	3.04	10.55	28.8
Cysteine	1.10	0.44	—
Glutamic acid	7.82	11.25	69.5
Glycine	2.37	3.11	76.2
Histidine	1.60	2.05	78.0
Isoleucine	3.45	4.19	82.3
Leucine	5.03	6.55	76.8
Lysine	3.15	3.91	80.6
Methionine	0.87	0.83	104.8
Phenylalanine	3.98	4.88	81.6
Proline	1.88	3.76	50.0
Serine	4.99	5.01[a)]	99.6
Threonine	2.44	2.82[a)]	86.5
Tryptophan	0.80	0.00	—
Tyrosine	3.05	3.00[a)]	101.6
Valine	3.13	4.10	76.3

a) A revised value for the errors resulting from decomposition of amino acid during acid hydrolysis, on the basis of the data reported by Hirs *et al.* (*11*).

1) Ten mg. of *Streptomyces griseus* protease containing 0.1 *PU* of proteinase activity was mixed with 20 ml. of 10% casein solution and allowed to react for 72 hours at pH 7.4, at 35°.

2) One volume of 10% casein solution was mixed with 40 volumes of 6*N* HCl and hydrolyzed at 115° for 24 hours in an air-tight tube.

develop many new industrial and scientific applications, in cooperation with the success in commercial production of this enzyme*.

1. Since the protease is capable of hydrolysing proteins until the majority of constituent amino acids are liberated as respective free amino acids, the protease is effective for preparing amino acid mixtures. In comparison with the usual acid hydrolysis, this method has a number of advantages, *e.g.*, the simplicity in equipment, the easiness in operation, the needlessness of deacidification and decolorization, the good qualities of the product, *etc.*

When a purified protein is used as a substrate, a clear hydrolysate is easily obtainable containing therein a remarkable amount of amino acids of high nutritive value such as tryptophan, methionine, threonine, lysine, *etc.*, as shown in Table IV. In addition to the above, the product was found to be superior to other similar amino acid mixture preparations in its purity; that is, in free amino acid content, odor, lack of color, anti-hygroscopic, *etc.* and so it can be utilized directly as eutrophics, drugs, foods and condiments. The hydrolysate prepared from impure protein such as animal intestines and other sources is also utilized as animal feed and bacterial medium. The hydrolysate of protein is also available as a raw material for isolating some valuable amino acids which are easily decomposed by acid hydrolysis.

2. The protease was also found to be useful for eliminating unnecessary proteinaceous impurities from animal- and plant-tissues. Applications in that field are being carried out in leather manufacture, silk degumming, drug manufacture, laundry, *etc.*

3. The protease is also available for improving the qualities of "miso" (*13*), bread, cookies, crackers, meat, and other foodstuffs.

4. In scientific investigation, *Streptomyces griseus* protease is greatly expected to offer a new experimental means for elucidating protein structure such as amino acid sequence or amino acid composition of proteins. For example, amino acid sequence of demoglobin and of Taka-amylase A were studied by Sasakawa *et al.* (*14*, *15*) and by Ikenaka (*16*) and Tsugita *et al.* (*17*), respectively, with the aid of the present protease. The protease was also used for isolating amino acid-azo dye compounds from liver protein by Terayama *et al.* (*18*, *19*, *20*, *21*). Recently, Toda *et al.* (*22*) reported that a partially modified, active Taka-amylase A can be divided into a smaller fragment which contains the original activity, by a hydrolysis with *Streptomyces griseus* protease. Their method seems to be available for studying active groups of enzymes. The protease was also used for isolating a diketopiperazine from gelatin by Koaze *et al.* (*23*).

SUMMARY

Substrate specificity of *Streptomyces griseus* protease has been ivestigated

* Partially purified *Streptomyces griseus* protease is put on the market from the Kaken Chemical Co., Ltd., in the trademark of "PRONASE".

by the following three methods; 1) Analysis of hydrolysing course of protein, 2) Assay for extent of hydrolysis of protein, 3) Quantitative analysis of amino acids liberated from protein.

It was found that the protease had an extremely broad substrate specificity and was capable of hydrolysing almost all peptide-bonds in proteins, until the majority of amino acids constructing the protein were liberated as the respective free amino acids. Therefore, the extent of hydrolysis of proteins was estimated to reach upward of 70–90 per cent in contrast to acid hydrolysis. So far as is known in the literatare, there is nothing as strong as the present protease. It is felt that the protease will help to develop many new industrial and scientific techniques.

A suitable method for preparing amino acid mixtures by this protease is described in this paper, together with some other applications of this protease.

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STUDIES ON TAKA-ACYLASE

I. PURIFICATION AND PROPERTIES OF TAKA-ACYLASE FROM TAKA-DIASTASE

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Acylase which hydrolyzes acyl amino acids is known to be a metal enzyme, activated by Co^{++}.

Greenstein *et al.* first studied acylase from hog kidney (*1-4*). Since then a considerable number of reports on acylase have been published mostly on the enzymatic resolution of racemic amino acids making use of the specificity of acylase. Acylases from molds have also been investigated by several workers (*5-7*). Recently Hata *et al.* (*8*) worked on acylase from the culture medium of *Aspergillus oryzae* and presented a model of the active site of the enzyme which consists of an enzyme-metal-substrate complex.

The present authors purified Taka-acylase from a fraction obtained during the crystallization of Taka-amylase A from taka-diastase (*9*). Some properties of the enzyme were elucidated.

MATERIALS AND METHODS

Substrates for Enzyme Assay—Acetyl-DL-phenylalanine (m.p. 145°) and chloroacetyl-DL-valine (m.p. 130°) were prepared by Schotten-Baumann reaction.

Measurement of Acylase Activity—Acylase activity was estimated from the librated amino acids by colorimetric ninhydrin method of Moore and Stein (*10*). The usual assay system consisted of 0.3 ml. of the enzyme solution, 0.4 ml. of 0.1 *M* veronal-HCl buffer of pH 7.8, and 0.3 ml. of 0.02 *M* acetyl-DL-phenylalanine or chloroacetyl-DL-valine solution. The reaction mixture was mixed with the activator, $CoCl_2$, to a final concentration of 5×10^{-4} *M*. Usually the reaction was started by adding the substrate. When the assay was conducted at higher pH's, borate buffer was used. After 15 minutes incubation at 37°, 1 ml. of ninhydrin solution* was added to the reaction mixture to stop the enzymatic action and the mixture was heated for 15 minutes in a boiling water bath. After cooling, the reaction mixture was diluted with 5 ml. of 50 per cent alcohol and the optical density of the solution was measured spectrophotometrically at 570 mμ in a 10 mm. light path.

The enzyme activity was usually expressed as the optical density, measured as mentioned above, and the specific acylase activity was expressed in units per mg. protein

* This consisted of 250 ml. of 4 *M* acetate buffer, pH 5.5, 750 ml. of methyl-cellosolve, 3 g. of hydrindantin and 2 g. of ninhydrin (*10*).

nitrogen (U/P.N.) or units per optical density at 280 mμ (U/E_{280}), where one unit of acylase was defined as the amount of enzyme which hydrolyzes one micromole of acetyl-DL-phenylalanine in 15 minutes under the conditions mentioned above.

Protein nitrogen was estimated by the micro-Kjeldahl method.

Measurement of Amylase Activity—Amylase activity was determined by Fuwa's method (*11*).

RESULTS AND DISCUSSION

Purification of Taka-Acylase—The starting material for the purification of Taka-acylase was the first rivanol precipitate usually discarded in the puri-

SCHEME 1

Purification of Taka-Acylase

Taka-diastase 2 kg.

↓ extraction with 10 liters of water, filtration

Filtrate -------- I

↓ addition of 0.25 *M* Ca-acetate to an equal volume of the filtrate, centrifugation

Supernatant

↓ 0.75 saturation with $(NH_4)_2SO_4$,

Precipitate

↓ dissolution in water and dialysis against running water overnight

Dialyzed Solution -------- II

↓ addition of 1 % rivanol solution (0.04 volume of the solution), filtration

Filtrate → Crystalline Taka-amylase A

Precipitate

↓ dissolution in 1 liter of 2 *M* sodium acetate, addition of 200 g. of Japanese acid clay, filtration

Filtrate -------- III

↓ addition of $CoCl_2$ to 10^{-3} *M*, heat treatment at 70° for 15 minutes, cooled, filtration

Filtrate

↓ dialysis against running water, addition of acetone to 50 % concentration, centrifugation

Precipitate

↓ dissolution in 0.1 *M* veronal-HCl buffer of pH 7.8 -------- IV zone electrophoresis

Purified Taka-acylase

fication of Taka-amylase A. One liter of 2 *M* sodium acetate was added to the precipitate per 2 kg. of taka-diastase and the former was dissolved. Then about 200 g. of Japanese acid clay was added to decolorize the solu-

tion and the mixture was filtered. $CoCl_2$ was added to the filtrate to a final concentration of $10^{-3}\,M$ and the solution was heated for 15 minutes at 70°. The precipitate formed was removed by filtration. By this heat treatment, almost all the amylase activity was eliminated, as shown in Table I. The filtrate was dialyzed against running water overnight in a cold room and the enzyme was precipitated by adding acetone (50 per cent). The precipitate collected by centrifugation was dissolved in a small amount of $0.1\,M$ veronal-HCl buffer, pH 7.8. Thus Taka-acylase was purified *ca.* 75 fold over the crude extract (I in Scheme 1), with a yield of 38 per cent. For further purification, the enzyme solution thus obtained was subjected to starch column-zone electrophoresis.

The purification procedure is shown in Scheme 1 and Table I gives a summary of the purification in terms of recovery and the specific activity. Table I also shows the specific activity and the residual activity of Taka-amylase A which is the main constituent of taka-diastase.

TABLE I

Total and Specific Activity and Yield at Each Step of Purification

Fraction number in Scheme 1	Acylase[1]				Amylase		Acylase/amylase
	Total units	Yield (%)	U/P.N.	U/E_{280}	Residual act. (%)	Specific[2] act.	
I	218,000	100	8.23	0.256	100	1	1
II	205,000	94	10.7	0.374	51.8	0.8	1.8
III	113,000	52	116.	5.95	3.26	0.9	15.9
IV	85,000	38	615.	30.0	0.017	0.04	2200.

1) Acetyl-DL-phenylalanine was used as substrate.
2) Arbitrary units/protein N.

Electrophoresis—Before electrophoresis, the enzyme solution was heated once more under the above conditions. After filtration, the enzyme solution was concentrated to about 30 ml. by adding acetone. Ten ml. of this solution of specific activity 80 U/E_{280} was then subjected to vertical starch column electrophoresis as follows; a column (4.5×62 cm.) of potato starch was used with a current of 40 mA. at 300 V. in veronal-HCl buffer solution containing $0.05\,M$ NaCl (ionic strength=0.1) pH 7.8 for 60 hours at 10°. After electrophoresis, the column was transferred to a fraction collector, eluted with the same buffer and 5 ml. fractions of effluent were collected. Protein concentration (optical density at 280 mμ) and acylase and amylase activities were determined. The results are shown in Fig. 1 and Table II. As shown in Fig. 1, protein was separated into two fractions (No. 10–54 and 70–98) and the acylase activity was found at the anode side of the main fraction (No. 31–40). Contaminating amylase activity was also found

in nearly the same fraction but judging from the results in Fig. 1, Taka-acylase seems to be a little acidic than Taka-amylase A. Fraction No. 35 which had the highest acylase activity contained enzyme *ca.* 6 fold purified over the solution used for electrophoresis or 1500 fold over the original crude extract (I in Scheme 1). Fractions No. 32 to 40 were collected and

TABLE II

Specific Activity and Yield of Taka-Acylase in the Fractions Shown in Fig. 1

	Yield (%)	Specific activity[1] (U/E_{280})
Enzyme solution used for electrophoresis	100.	80
Fraction No. 33	30.0	351
No. 35	35.0	488
No. 37	13.5	288
No. 32 to No. 40	93.0	312

1) Acetyl-DL-phenylalanine was used as substrate.

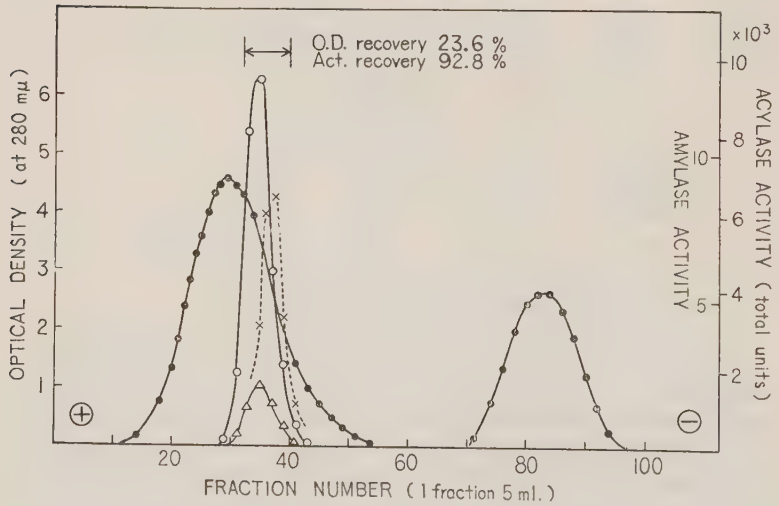

FIG. 1. Starch column-zone electrophoresis of partially purified Taka-acylase.

—●—: absorption at 280 mμ —○—: acylase activity measured with acetyl-DL-phenylalanine as substrate —△—: acylase activity measured with chloroacetyl-DL-valine as substrate —×—: amylase activity.

the recovery of protein (optical density) and acylase activity of these fractions were 23.6 and 92.8 per cent respectively; therefore the enzyme was purified *ca.* 4 fold over the solution used for electrophoresis or 1000 fold

over the crude extract solution.

To see whether the same enzyme hydrolyzed both aromatic and aliphatic substrates, acylase activity was measured with acetyl-DL-phenylalanine and chloroacetyl-DL-valine. It can be seen in Fig. 1 that the peak of acylase activity measured with chloroacetyl-DL-valine closely coincides with the peak with acetyl-DL-phenylalanine.

PROPERTIES

pH-Activity Curve—The pH-activity curve was measured with various concentrations of Co^{++}, using enzyme which had been dialyzed against deionized water at 5° for about 30 hours. Fig. 2 and 3 show results using acetyl-DL-phenylalanine and chloroacetyl-DL-valine as substrate respectively. It is shown in Fig. 2 that the pH optimum depends on the concentration of Co^{++} in the reaction mixture. At a low concentration of Co^{++}, the pH-activity curve is a normal bell-shape one having an optimum at pH 8.6, whereas at higher concentrations (higher than 10^{-4} *M* Co^{++}) two pH optima were obtained at 8.0 and 10.0. The pH optimum at 8.0 agrees with the results obtained by other investigators (*5–8*). Similar results were

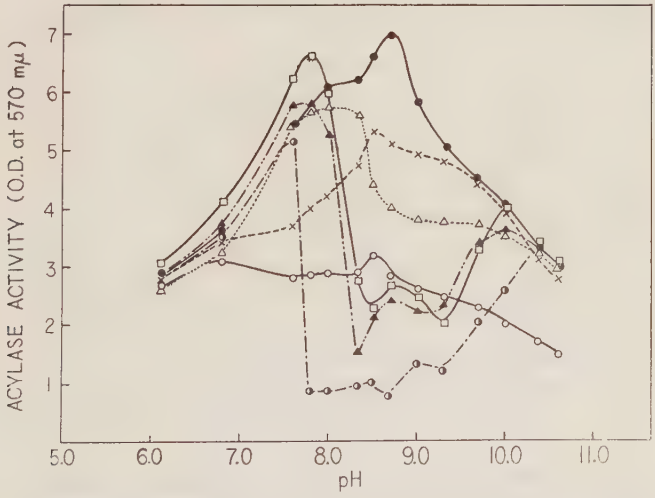

FIG. 2. pH-activity curves of Taka-acylase using acetyl-DL-phenylalanine as substrate with or without added Co^{++}. The assay system was as described in the text except that borate buffer was used. The enzyme used was dialyzed against deionized water at 5° for about 30 hours.
—○— 0 ---×--- 1×10^{-5} —●— 1×10^{-4} ··△·· 2.5×10^{-4}
—□— 5.0×10^{-4} —··▲—·· 7.5×10^{-4} —·—◑—·— 2.5×10^{-3} *M* Co^{++}.

obtained using chloroacetyl-DL-valine, as shown in Fig. 3. But in this case maximal activity was obtained at pH 7.5 in the presence of 10^{-3} *M* of Co^{++}.

The fact that two pH optima were obtained could be explained as

follows: as shown in Fig. 4, a considerable portion of enzyme activity was

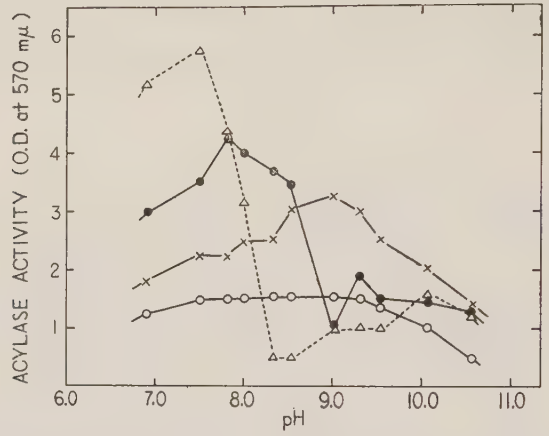

Fig. 3. pH-activity curves of Taka-acylase using chloroacetyl-DL-valine as substrate, with or without added Co^{++}. The assay system and the enzyme used were as described in Fig. 2.
—○— 0 —×— 10^{-5} —●— 10^{-4} --△-- 10^{-3} *M* Co^{++}

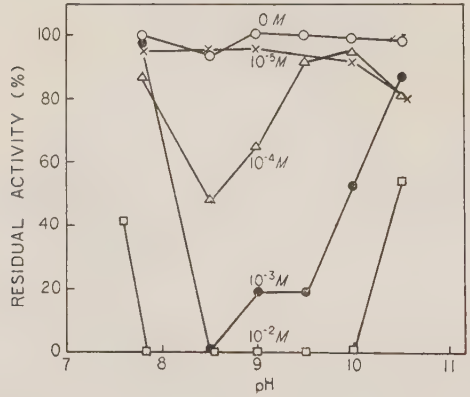

Fig. 4. Residual activity after filtration of the cobaltous precipitate formed in the enzyme-Co^{++} solution at various pH's. One ml. of the enzyme solution and 0.8 ml. of borate buffer solution of each pH were mixed and to the mixture 0.2 ml. of Co^{++} solution was added to final concentrations indicated. The mixture was incubated for 30 minutes at 30°. After incubation, the precipitate was filtered off and the filtrate diluted with veronal-HCl buffer of pH 7.8. The residual activity in the solution was measured as described in the text, using acetyl-DL-phenylalanine as substrate.

eliminated after filtration of the cobaltous precipitate, which was formed in the presence of higher concentrations of Co^{++} at between pH 8.0 and

10.0. This suggests that the enzyme was adsorbed on the cobaltous precipitate, thus reducing the enzyme activity in the high pH range and thus two pH optima were obtained at higher concentrations of Co^{++}, as shown in Fig. 2 and 3. Therefore the true optimum pH seems to be at 8.6, at least with acetyl-DL-phenylanine as substrate. It may be possible to apply this phenomenon to the purification of the enzyme.

pH Stability—The effect of pH on the stability was investigated at 37° for 2.5 and 13 hours and at 60° for 15 minutes. The results are shown in Fig. 5. It was found that the enzyme is stable at between pH 8.0 and 9.0. The same results were obtained with both aromatic and aliphatic substrates.

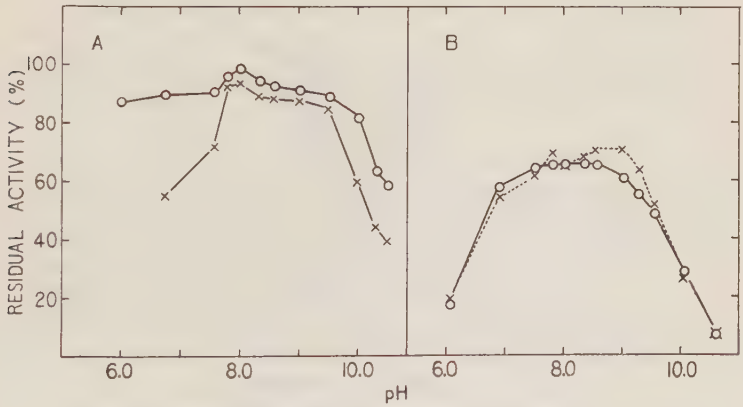

FIG. 5. Effect of pH on the stability of Taka-acylase. Incubations were in borate buffer of various pH's. After incubation, the enzyme was diluted with veronal-HCl buffer, pH 7.8. The enzyme was assayed as described in the text.

A: incubated at 37° for 2.5 (—○—) and 13 hours (—×—).

B: incubated at 60° for 15 minutes.

For the assay of the residual activity, acetyl-DL-phenylalanine (—○—) and chloroacetyl-DL-valine (---×---) were used as substrate.

Effect of Various Metal Ions—The effects of various metal ions are shown in Fig. 6. It can be seen that only Co^{++} activates the enzyme and the other metals are inhibitory. The optimal concentration of Co^{++} was from 10^{-3} to 10^{-4} *M* at pH 7.8. Besides the metal ions shown in Fig. 6, Ca^{++}, Sr^{++} and Mg^{++} were also tested but these had no effect even at a concentration of 10^{-2} *M*. From the results in Fig. 6, the order of 50 per cent inhibition is as follows: $Cu^{++} > Cd^{++} > Fe^{++} > Cr^{+++} > Fe^{+++} > Ni^{++} > Zn^{++} > Mn^{++}$.

When ethylenediamine tetracetate (EDTA) was added to the reaction mixture at a concentration of 10^{-4} *M*, almost all the activity was lost but by adding Co^{++} (more than 10^{-4} *M*) the activity was restored.

Heat Stability—Taka-acylase is very heat stable under the conditions mentioned in the "purification". H a t a *et al.* (*8*) reported that the enzyme

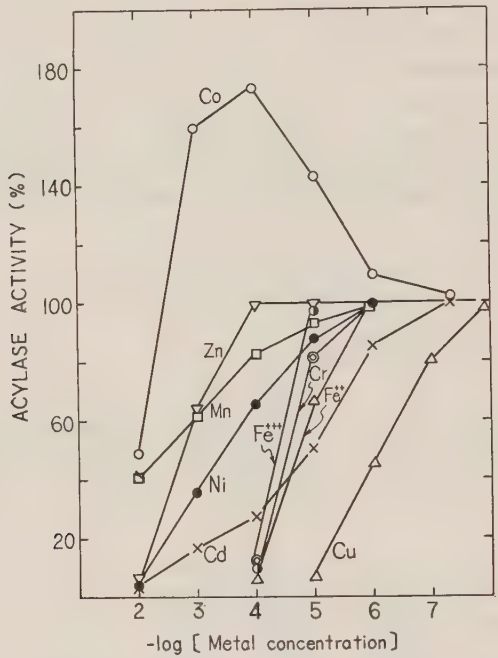

Fig. 6. Effect of various metal ions on Taka-acylase activity. The enzyme assay was performed as described in the text except that various metal ions were added during incubation.

was heat stable in the presence of both Co^{++} and certain anions (acetate and sulfate). The authors also investigated the effect of metal ions and anions on the heat stability. As shown in Fig. 7, Taka-acylase is very heat stable in the presence of both sodium acetate (1.5 *M*) and Co^{++} (1.3×10^{-2} *M*), while Taka-amylase A is rapidly inactivated at over 40°, irrespective of the presence of the above two factors. When sodium sulfate was used instead of sodium acetate, it was also found to have a protective effect in the presence of Co^{++}, but sodium chloride and nitrate did not show any such effect at a concentration of 2 *M*. The effect of the concentrations of Co^{++} and anions (acetate and sulfate) on the heat stability, is shown in Fig. 8 and 9. These results show that Taka-acylase is heat stable in the presence of 10^{-3} *M* Co^{++} and acetate or sulfate at a concentration of more than 1 *M*. Table III shows the effect of various metal ions on heat stability in the presence of 1.5 *M* sodium acetate (pH 7.8). Since, as mentioned above, the metal ions used inhibit the acylase action, the enzyme assay was carried out after the metal ion had been removed with EDTA. Experimental

details are described in Table III. The enzyme is protected from heat denaturation not only by Co^{++} but also by metal ions which act as inhi-

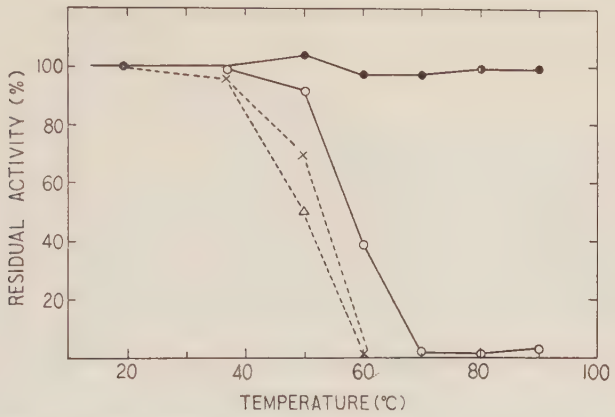

FIG. 7. Heat stability of Taka-acylase and Taka-amylase A in the presence of Co^{++} and/or sodium acetate. The final concentration of Co^{++} and of acetate were $1.3\times10^{-2}M$ and $1.5M$ (at pH 7.8) respectively. After 15 minutes incubation at each temperature the enzyme was assayed as described in the text. Control assays were carried out for each experiment.

Acylase {—●—: Co^{++} and AcONa
Acylase {—○—: Co^{++} only or AcONa only
Acylase {---×---: AcONa
Acylase {---△---: Co^{++} and AcONa

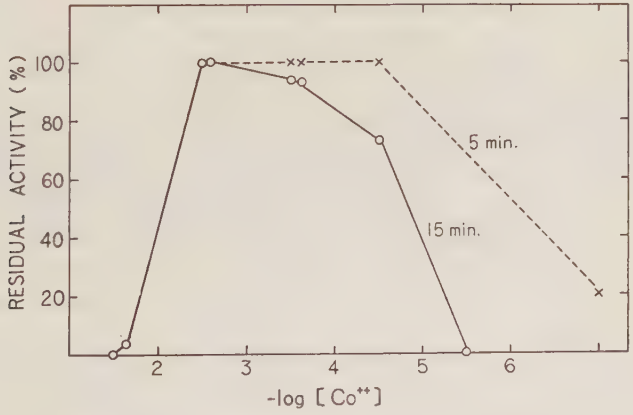

FIG. 8. Effect of the concentration of Co^{++} on the heat stability of Taka-acylase in the presence of sodium acetate. The enzyme solution in $1.4M$ sodium acetate was incubated for 5 or 15 minutes at 80° (pH 7.8). After incubation, the residual activity was measured as described in the text. Control assays were carried out at each concentration of Co^{++}. Control values were taken as 100 per cent activity.

bitors for the enzyme action. In other words, metal ions which have an affinity for the enzyme protein, acting either as activators or inhibitors,

can protect Taka-acylase from heat denaturation when adequate anions are present. The effect of the anions on enzyme denaturation is similar to

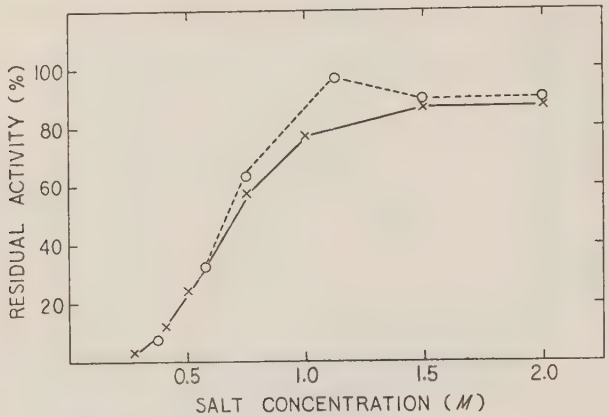

FIG. 9. Effect of the concentration of sodium acetate and sulfate on the heat stability of Taka-acylase in the presence of $5\times10^{-4}\,M$ of Co^{++}. The experiments were performed as described in Fig. 8 except that the enzyme solution was incubated in various concentrations of sodium acetate and sulfate in the presence of $5\times10^{-4}\,M$ of Co^{++} (pH 7.8). ---○--- Na_2SO_4, —×— AcONa

TABLE III

The Effect of Various Metal Ions on the Heat Stability of Taka-Acylase

The enzyme solution containing $1.5\,M$ sodium acetate and $1.3\times10^{-3}\,M$ metal ion was incubated for 10 minutes at 70° at pH 7.8. The enzyme assay was carried out as follows; to 0.3 ml. of the enzyme solution ($1.3\times10^{-3}\,M$ metal ion), 0.3 ml. of $2\times10^{-3}\,M$ EDTA solution was added and the mixture was incubated at room temperature. Then 0.3 ml. of veronal-HCl buffer (pH 7.8) containing $10^{-2}\,M$ Co^{++} was added. The reaction was started by adding 0.3 ml. of $0.02\,M$ acetyl-DL-phenylalanine. Control experiments were performed with each metal ion.

metal ion:	Co^{++}	Mn^{++}	Fe^{++}	Cd^{++}	Zn^{++}	Hg^{++}	Ni^{++}	Mg^{++}	Ca^{++}	none
residual activity (%)	100	100	100	94.5	96.0	75.6	59.6	10.1	10.0	8.4

their effect on some proteins as observed by Greenstein (*12*) and Burk (*13*). To elucidate this effect, further investigations must be undertaken.

Effect of p-Chloromercuribenzoate (PCMB)—Hata *et al.* (*8*) reported that acylase activity was not inhibited by PCMB. Using dialyzed enzyme, PCMB inhibited the acylase activity by 48 per cent at a concentration of $3.6\times10^{-4}\,M$ as shown in Table IV and the activity was restored by the addition of thioglycolate. From these results Taka-acylase seems to be an

SH-enzyme.

Comparison of Taka-Acylase with Pancreas Carboxypeptidase and Hog Kidney Acylase I—To see whether the enzyme has carboxypeptidase (CPase) action, the activity of Taka-acylase was compared with that of crystalline pancreas CPase* using several substrates *i.e.*, acetyl-L-phenylalanine, chloroacetyl-L-

Table IV

Inhibition of the Acylase Activity by PCMB and Reversal of Inhibition by Thioglycolate

The enzyme assay was carried out at pH 8.6 under the conditions shown in the table. When thioglycolate was used, it was added to the reaction mixture after the addition of PCMB. Acetyl-DL-phenylalanine was used as substrate. The enzyme used was dialyzed against deionized water for 40 hours at 5° before used.

conc. of PCMB / Reagent	0	$1.4\times10^{-4}\,M$[1]		$3.6\times10^{-4}\,M$[1]	
PCMB	−	+	+	+	+
Thioglycolate $2.1\times10^{-5}\,M$[1]	−	+	−	+	−
Residual act. (%)	100	93	73	77	52

1) Final concentration of the reaction mixture.

Table V

Comparison of Taka-Acylase with Pancreas Carboxypeptidase and Hog Kidney Acylase I

The enzyme assay for Taka-acylase and CPase was carried out at pH 8.6 in borate buffer. For Taka-acylase the reaction mixture contained $10^{-5}\,M$ Co^{++}. The final concentration of each substrate was $3\times10^{-3}\,M$. The values for acylase I are cited from data of Greenstein *et al.* (*1*).

Substrate:	Ac-Phe	ClAc-Phe	ClAc-Tyr	ClAc-Val	Cbz-Gly-Leu
Taka-acylase	1[a]	0.575	0.475	0.153	0
Carboxypeptidase	1	366.	236.	4.81	364.
Acylase I	1	32.6	23.2	35.0	—

a) The values are calculated as units of the activity. Ac-Phe; acetyl-L-phenylalanine, ClAc-Phe; chloroacetyl-L-phenylalanine, ClAc-Tyr; chloroacetyl-L-tyrosine, ClAc-Val; chloroacetyl-L-valine, Cbz-Gly-Leu; carbobenzoxyglycyl-L-leucine.

phenylalanine, chloroacetyl-L-tyrosine, chloroacetyl-L-valine and carbo-

* Purchased from Worthington Biochemical Corp.

benzoxy-glycyl-L-leucine. Table V shows the relative susceptibility of these substrates to both enzymes. It was found that Taka-acylase did not attack carbobenzoxyglycyl-L-leucine, the most susceptible substrate for CPase. Replacement of the acetyl group of the substrate by a chloroacetyl group does not increase the susceptibility to Taka-acylase, in contrast to the effect on CPase. In Table V there are also listed the susceptibilities of the substrates used above to hog kidney acylase-I as cited from data of Greenstein *et al.* (*1*).

Identity of the Acylase Hydrolyzing Aromatic and Aliphatic Substrates—Birnbaum (*14*) suggested that besides acylase I and II there is another enzyme, acylase III, in hog kidney which specifically hydrolyzes aromatic substrates. Purified Taka-acylase hydrolyzes both aromatic and aliphatic substrates and, as already mentioned in the section on "Electrophoresis", it was suggested from electrophoretic results that both substrates were hydrolyzed by the same enzyme. This can also be seen from the behavior in the course of heat denaturation, as shown in Table VI. During heat denaturation, the ratio of the activity measured with acetyl-DL-phenylalanine to that with chloroacetyl-DL-valine is constant. Furthermore the pH-stability curve as measured with either substrate (see Fig. 5-B) is the same. Therefore both substrates seem to be hydrolyzed by a single enzyme. This will be further discussed in connection with kinetic results, in the next paper.

TABLE VI

The Ratio of the Activity with Acetyl-DL-Phenylalanine to That with Chloroacetyl-DL-Valine during Heat Denaturation

The enzyme solution of pH 8.6 in borate buffer was incubated for 15 minutes at each temperature. The residual activities were measured as described in the text with acetyl-DL-phenylalanine and chloroacetyl-DL-valine as substrates.

Temperature		20°	37°	50°	60°	70°	80°
Residual Activity (%)	Ac-Phe	98.	100.	84.	46.	3.3	0
	ClAc-Val	102.	100.	83.5	48.	3.4	0
Ac-Phe/ClAc-Val		0.96	1.00	1.01	0.94	0.95	—

Ac-Phe; acetyl-DL-phenylalanine, ClAc-Val; chloroacetyl-DL-valine.

Activation Energy—Activation energies were measured by means of Arrhenius' plot using various substrates, *i.e.*, acetyl-L-phenylalanine, chloroacetyl-L-phenylalanine, -L-tyrosine and -L-valine. As shown in Fig. 10, chloroacetyl-L-valine at above 22°, acetyl-L-phenylalanine at below 22°, chloroacetyl-L-phenylalanine and chloroacetyl-L-tyrosine gave nearly the

same activation energy, *ca.* 14000 cal. These results were obtained in the presence of a constant concentration of Co^{++}. The effect of the Co^{++} concentration on the activation energy is now being investigated.

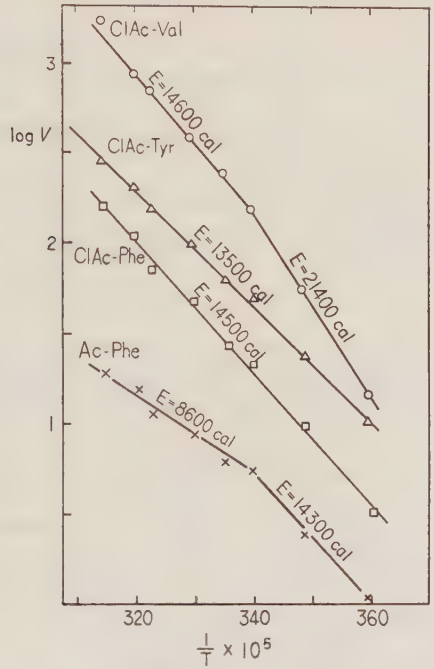

FIG. 10. Arrhenius' plots for the Taka-acylase reaction using various substrates at pH 7.8. The enzyme assay was carried out at pH 7.8 at a concentration of 4.5×10^{-4} *M* of Co^{++} and the assay system was as described in the text. Substrates were used at a final concentration of 3×10^{-3} *M*. Following abbreviations were used: ClAc-Val; chloroacetyl-L-valine, ClAc-Tyr; chloroacetyl-L-tyrosine, ClAc-Phe; chloroacetyl-L-phenylalanine, Ac-Phe; acetyl-L-phenylalanine.

SUMMARY

Taka-acylase was purified *ca.* 1000 fold from taka-diastase by starch column-zone electrophoresis.

The enzyme is activated only by Co^{++} and various other metal ions are inhibitory. It was found that both Co^{++} and metal ions having an inhibitory action protected the enzyme from heat denaturation when they were present together with sodium acetate or sulfate.

A pH optimum was found at 8.6 at low concentrations of Co^{++}. At higher concentrations of Co^{++} two pH optima were observed at 8.0 and 10.0.

Substrate specificity of Taka-acylase was compared with that of pan-

creas carboxypeptidase.

Some other properties of the enzyme were investigated and discussed.

The authors wish to express their gratitude to Dr. A. Tsugita for his valuable advice throughout this investigation and also to Sankyo Co., Ltd. for kindly supplying "Taka-diastase Sankyo".

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CONDENSATION REACTION OF DOPA WITH ETHYLENEDIAMINE

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In the previous papers (*1*, *2*, *3*), the separation of the condensation products of ethylenediamine with various catechol derivatives of biological importance was reported, and the properties of the main condensates were described.

The main condensate of noradrenaline with ethylenediamine was found to be identical with that of catechol (*2*). By this result, discussions raised by Joly and Nadeau (*4*) on our first report (*1*) was clearly dissolved.

During this series of studies, the condensate of DOPA with ethylenediamine was also examined, and it was reported that it took about 2 hours to obtain the stable fluorescence of the condensation product (*3*), however, the precise study of the reaction has not been practiced. As the biochemical meaning of DOPA has recently been noted, the condensation reaction of DOPA with ethylenediamine, which may be useful for the micro-analysis of DOPA, was examined in detail.

METHODS

To 100 μg. of DL-DOPA in 5 ml. of water, 0.25 ml. of 2 *M* ethylenediamine dihydrochloride and 0.35 ml. of ethylenediamine base (anhydrous) were added (*5*). The reaction mixture was heated at 50°, and the change of the fluorescence spectrum of the reaction mixture was observed at intervals during the incubation. The fluorescence spectra of the reaction mixture were recorded by a fluorospectrophotometer designed by Yagi *et al.* (*6*). The condensation products were excited by ultraviolet ray of 365 mμ.

The change of the absorption spectrum of the reaction mixture was measured using 5 times concentrated DL-DOPA solution treated in the same way as above. The absorption spectrum was recorded by a Beckman DK-2 spectrophotometer at the same intervals as those in the recording of fluorescence spectrum. A reagent blank, which was treated just in the same way as the main experiment, was used.

RESULTS

In a preliminary experiment, it was found that the colour of the fluorescence observed under ultraviolet light changed from blue to yellow

with the duration of reaction.

The fluorescence spectrum recorded at each reaction time is reproduced in Fig. 1. Five minutes after the reaction, blue fluorescence (λ_{max} at 465 mμ) was observed. The fluorescence intensity gradually increased and the fluorescence peak shifted to the longer wavelength. After 40 minutes, the fluorescence intensity decreased and the fluorescence peak moved continuously till 90 minutes, when the fluorescence intensity became constant (λ_{max} at 555 mμ).

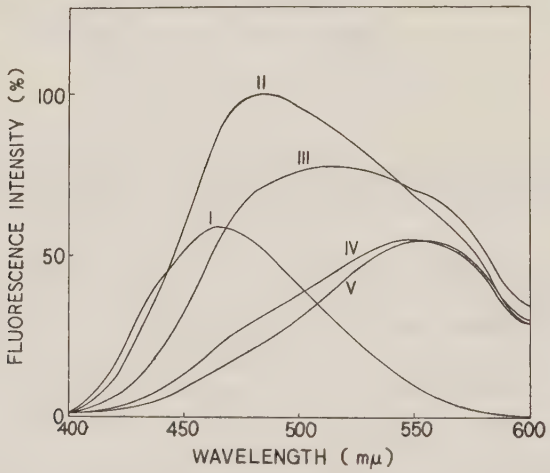

Fig. 1. Change of fluorescence spectrum of the reaction mixture of DOPA (18 μg./ml. in final concentration) and ethylenediamine. The reaction mixture was heated at 50°, and the fluorescence spectrum was recorded at different reaction times: I, after 5 min.; II, after 20 min.; III, after 40 min.; IV, after 60 min.; V, after 90 min. The reaction mixture was excited by ultraviolet ray of 365 mμ. Fluorescence spectrum was measured by the fluorospectrophotometer.

The change of the absorption spectrum of the reaction mixture was also examined at different reaction times as those in the recording of fluorescence spectrum. The results are shown in Fig. 2. Five minutes after the reaction, two absorption peaks at 237 mμ and 297 mμ were observed. By further heating, the other two peaks at 258 mμ and 405 mμ appeared. After 40 minutes the third peak appeared at 350 mμ as a shoulder. After 90 minutes the absorption spectrum became constant, and had three marked peaks at 258 mμ, 350 mμ and 405 mμ.

These results suggest that the condensate obtained in the present experimental condition had not been derived by a simple step of the reaction. The first step is the development of a substance which was shown by curve I in Fig. 1 and 2. It gives rise to another substance which has an

fluorescence maximum at 555 mμ and the absorption spectrum shown by curve V in Fig. 2.

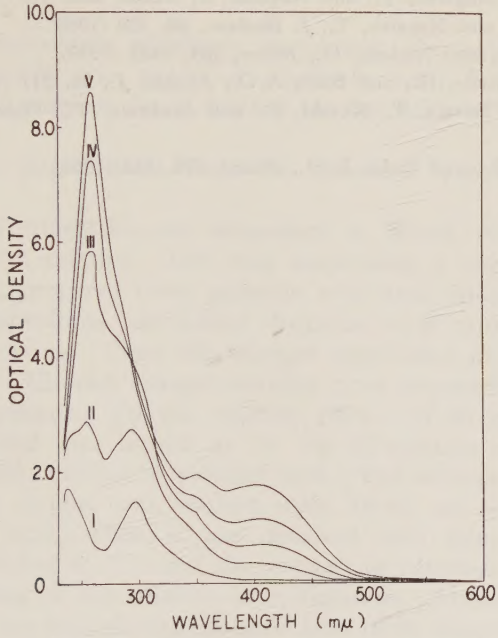

FIG. 2. Change of absorption spectrum of the reaction mixture of DOPA (90 μg./ml. in final concentration) and ethylenediamine. The reaction mixture was heated at 50°, and the absorption spectrum was recorded at the following time of reaction. I, after 5 min; II, after 20 min.; III, after 40 min.; IV, after 60 min.; V, after 90 min. A reagent blank, which was treated in the same way as the main experiment, was used.

These results are in accordance with those reported by Burn *et al.* (*7*) that the reaction of noradrenaline with ethylenediamine proceeds in at least two steps. It was revealed from these experiments that in the fluorimetric analysis of DOPA by its ethylenediamine condensation, long time incubation (more than 90 minutes at 50°) is required.

SUMMARY

The dynamic changes of the fluorescence spectrum and of the absorption spectrum during the condensation reaction of DOPA and ethylenediamine were measured. The results indicate that the reaction proceeds in at least two steps and that more than 90 minutes of reaction at 50° is required to obtain a constant fluorescent product.

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CHEMICAL SYNTHESIS OF RIBOFLAVIN PALMITATE

To obtain fat-soluble riboflavin derivatives, the authors intended to synthesize chemically the esters of riboflavin with fatty acids. These substances are supposed to be of interest in the fields of biochemistry and nutrition.

The present paper deals with the chemical synthesis of riboflavin palmitate.

One gram of riboflavin was suspended in 300 ml. of dry chloroform-pyridine (1 : 1, *v/v*) mixture. Into this suspension, a mixture of 15 g. of palmityl chloride, prepared from palmitic acid and thionyl chloride, and 50 ml. of dry chloroform was added dropwise at 0° within about 1 hour under vigorous stirring. Then, the mixture was shaken at 33° for 14 hours. The transparent yellowish orange solution thus obtained was evaporated under reduced pressure. To the residue, 100 ml. of 90 per cent aqueous pyridine was added and heated at 70° for 15 minutes to decompose the remaining palmityl chloride to palmitic acid. The solution was evaporated completely. The residue was washed with 200 ml. of methyl alcohol to remove palmitic acid. Then, it was dissolved with minimum volume of absolute ethyl alcohol at 75°, and the crystals of riboflavin palmitate were obtained by storing of this solution in refrigerator. About 500 mg. of pure riboflavin palmitate crystals was obtained after three times recrystallizations from absolute ethyl alcohol. It melts at 78.5°.

Then, chemical structure of riboflavin palmitate was studied as follows:

Riboflavin in the Sample—From the data of absorption spectrum, it was easily supposed that this substance contains isoalloxazine nucleus. To ascertain the above assumption, the saponification of the sample was practiced. When alcoholic solution of riboflavin palmitate was mixed with its equal volume of *N* NaOH, and stood at room temperature in the dark, it produced riboflavin, which was judged by paper chromatography.

Furthermore, it was found that the palmitate, in its diluted alcoholic solution, was converted quantitatively to lumiflavin by the procedure of the lumiflavin fluorescence method (*1*). Then, the riboflavin was analyzed by the lumiflavin fluorescence method and 138 μg. of it (calculated in the terms of riboflavin) was found in 500 μg. of the sample.

Palmitic Acid in the Sample—The quantity of palmitic acid in the sample was determined by the method of Entenman (*2*), and 385 μg. of it (calculated in the terms of palmitic acid) was found in 500 μg. of the sample. So, it is calculated that one mole of riboflavin combines with 4 moles of palmitic acid.

Consumption of Periodate of the Sample—From the above data, it was supposed that each of four molecules of palmitic acid might be combined at

the positions of 2′, 3′, 4′, 5′ carbons of ribitol chain of riboflavin, respectively. To ascertain this, the consumption of periodate (*3*) by the sample was tested in acetic acid at 0°. No consumption of periodate was observed even after 72 hours of the incubation. Thus, chemical structure of the synthesized sample was determined to be riboflavin 2′, 3′, 4′, 5′-tetrapalmitate.

Elemental Analysis—The following data of elemental analysis of the sample further supported the above-mentioned chemical structure.

	C%	H%	N%
Calculated for $C_{81}H_{140}O_{10}N_4$	73.15	10.61	4.21
Found	72.93	10.73	4.41

The riboflavin palmitate is easily dissolved in pyridine, ethyl alcohol, benzene, ethyl ether, and neutral fat, but not in water.

The detailed report of this communication will be published in the future.

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